สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

LITERATURE REVIEW

In living organisms, the most abundant substrate for most cells is the extracellular matrix or ECM. It adheres to cells via integrins, which are membranespanning heterodimetrip receptors (Hynes, 1999). Through the cell matrix adhesions, the ECM transduces physiological signals regulating cell growth, cell proliferation, cell differentiation, and matrix remodeling to the cells (Hynes, 1999). Therefore, the ECM plays an important role in living tissue development and regeneration.

Tissue regeneration is achieved by culturing isolated cells on biocompatible and biodegradable bioscaffolds which cells are seeded. A number of studies have shown the importance of selecting the appropriate biomaterials as bioscaffols for the cell adhesion and supporting the cell proliferation (Hutmacher, 2000). Given the reasons above, the ideal bioscaffold material should be one which closely mimic the natural environment in the tissue-specific ECM (Suh *et al.*, 2000).

2.1 Fabrication Techniques

So far there are a variety of different techniques used to produce porous structures for tissue engineering applications. Each technique has its own advantages and drawbacks.

2.1.1 Nanofiber Self-Assembly:

Molecular self-assembly is one of the few methods to create biomaterials with properties similar in scale and chemistry to that of the natural in vivo extracellular matrix (ECM). Moreover, these hydrogel bioscaffolds have shown superior in vivo toxicology and biocompatibility compared with traditional macroscaffolds and animal-derived materials.

2.1.2 Textile technologies:

These techniques include all the approaches that have been successfully employed for the preparation of non-woven meshes of different polymers. In particular non-woven polyglycolide structures have been tested for tissue engineering applications: such fibrous structures have been found useful to grow different types of cells. The principal drawbacks are related to the difficulties of obtaining high porosity and regular pore size.

2.1.3 Solvent Casting & Particulate Leaching (SCPL):

This approach allows the preparation of porous structures with regular porosity, but with a limited thickness. First the polymer is dissolved into a suitable organic solvent (e.g. polylactic acid could be dissolved into dichloromethane), then the solution is cast into a mold filled with porogen particles. Such porogen can be an inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres. The size of the porogen particles will affect the size of the scaffold pores, while the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure. After the polymer solution has been cast the solvent is allowed to fully evaporate, then the composite structure in the mold is immersed in a bath of a liquid suitable for dissolving the porogen: water in case of sodium chloride, saccharose and gelatin or an aliphatic solvent like hexane for paraffin. Once the porogen has been fully dissolved a porous structure is obtained. Other than the small thickness range that can be obtained, another drawback of SCPL lies in its use of organic solvents which must be fully removed to avoid any possible damage to the cells seeded on the scaffold.

2.1.4 Gas Foaming:

To overcome the necessity to use organic solvents and solid porogens a technique using gas as a porogen has been developed. First disc shaped structures made of the desired polymer are prepared by means of compression molding using a heated mold. The discs are then placed in a chamber where are exposed to high pressure CO_2 for several days. The pressure inside the chamber is gradually restored to atmospheric

levels. During this procedure the pores are formed by the carbon dioxide molecules that abandon the polymer, resulting in a sponge like structure. The main problems related to such a technique are caused by the excessive heat used during compression molding (which prohibits the incorporation of any temperature labile material into the polymer matrix) and by the fact that the pores do not form an interconnected structure.

2.1.5 Emulsification/Freeze-drying:

This technique does not require the use of a solid porogen like SCPL. First a synthetic polymer is dissolved into a suitable solvent (e.g. polylactic acid in dichloromethane) then water is added to the polymeric solution and the two liquids are mixed in order to obtain an emulsion. Before the two phases can separate, the emulsion is cast into a mold and quickly frozen by means of immersion into liquid nitrogen. The frozen emulsion is subsequently freeze-dried to remove the dispersed water and the solvent, thus leaving a solidified, porous polymeric structure. While emulsification and freeze-drying allows a faster preparation if compared to SCPL, since it does not require a time consuming leaching step, it still requires the use of solvents, moreover pore size is relatively small and porosity is often irregular. Freeze-drying by itself is also a commonly employed technique for the fabrication of scaffolds. In particular it is used to prepare collagen sponges: collagen is dissolved into acidic solutions of acetic acid or hydrochloric acid that are cast into a mold, frozen with liquid nitrogen then liophylized.

2.1.6 Thermally Induced Phase Separation (TIPS):

Similar to the previous technique, this phase separation procedure requires the use of a solvent with a low melting point that is easy to sublime. For example dioxane could be used to dissolve polylactic acid, then phase separation is induced through the addition of a small quantity of water: a polymer-rich and a polymer-poor phase are formed. Following cooling below the solvent melting point and some days of vacuum-drying to sublime the solvent a porous scaffold is obtained. Liquid-liquid phase separation presents the same drawbacks of emulsification/freeze-drying.

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2.1.7 CAD/CAM Technologies:

Since most of the above described approaches are limited when it comes to the control of porosity and pore size, computer assisted design and manufacturing techniques have been introduced to tissue engineering. First a three-dimensional structure is designed using CAD software then, the scaffold is realized by using ink-jet printing of polymer powders or through Fused Deposition Modeling of a polymer melt.

2.2 Electrospinning Technique

Polymer fibers ranging from 10 to 100 μm can be produced by conventional melt, dry, or wet spinning processes. Most commonly a polymer melt is extruded and subsequently drawn. For the generation of finer fibers, ranging from 15 nm to 10 μm or greater, electrospinning is a broadly useful technology. Electrospinning relies on the application of an electrostatic force to drive fiber formation. The fibers are often collected in a nonwoven mesh characterized by high surface area/unit mass. A large fraction of the volume of such non-woven meshes is in the form of interconnected porosity. Interest in the electrospinning technology was recently revived when Reneker et al. first demonstrated that a multitude of polymers could be electrospun (Reneker et al., 1996) and refined the associated theory (Reneker et al., 2000). Hundreds of different natural and synthetic compositions have been electrospun into thin fibers since 1990.

The electrospinning process itself is over 70 years old whereas the concept of electrospun scaffolding for biomedical applications appears to have first emerged in 1978. "An Elastomeric Vascular Prosthesis" was reportedly produced from polyurethane elastomer utilizing "electrostatic spinning" by Annis et al.

2.3 The electrospinning process

The elements required for electrospinning include a polymer source, a high voltage supply, and a collector (Fong et al., 1999). When an electric potential is applied between the polymer source and collector, charge accumulates and is forced to the surface of an emerging polymeric droplet at the end of a metal needle. In electrospinning, the force of the electric field overcomes the cohesive force of the solution, often dominated by surface tension, and an electrically charged jet of polymer-containing solution erupts. As the jet moves toward the collector plate, it is elongated by electrostatic interactions between charges on nearby segments of the same jet. Meanwhile, the solvent evaporates and finally the jet solidifies into a fiber. Typical electrospinning processes create very long fibers that can vary in diameter along the length from one half to as much as twice the average diameter. The diameter of these fibers may be considerably larger than the nanometer scale.

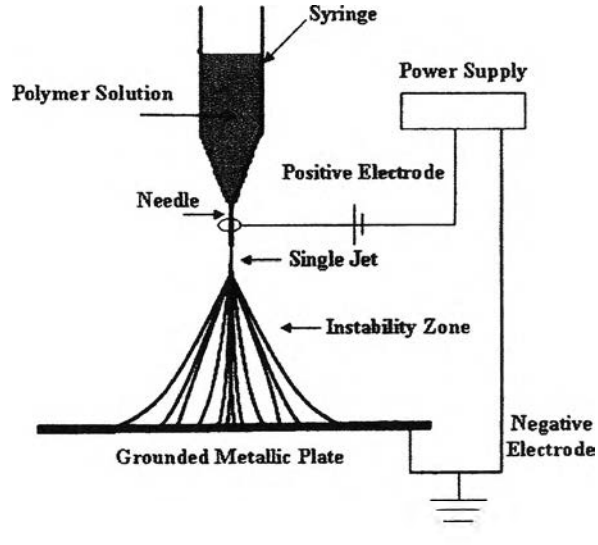

Figure 2.1 Electrospinning set up.(Keun-Hyung Lee et al., 2006)

2.4 Morphology

It is well known that cells are sensitive to the topography of the supporting surface although the exact reasons for this are unclear. In electrospinning, there are techniques that can be used to influence the nanoscale surface morphology of the fibers themselves (Casper *et al.*, 2004). A great variety of such nanoscale surfaces can be made to support cells. The search for surfaces having strong, well-defined beneficial influences on cell growth is an active subject of contemporary research. At a slightly larger scale (1 to 10 μm), the electrospinning process has an additional role in determining topography. Spindle-shaped beads or nearly spherical beads on the nanofibers may be produced. Solvent volatility is a factor: if the fluid jet is collected prior to complete solvent evaporation, the deformable fibers may either flatten upon impact with the surface of a collector or adhere to other fibers. If the arriving jet or fiber lands on previously collected fibers the still fluid material can merge and coalesce at crossing point to create a conglutinated network.

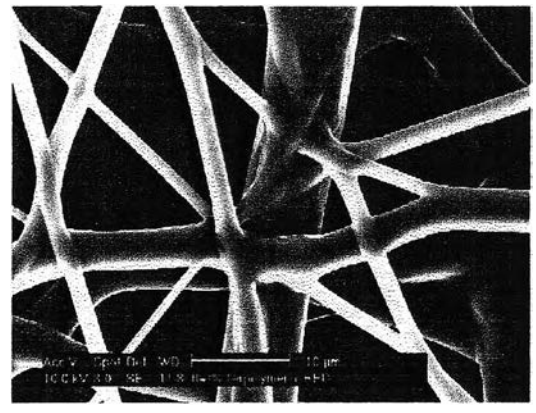

Figure 2.2 . Fibers containing high concentrations of solvent can form mechanical connections with other fibers at their crossing points. This creates a threedimensional (i.e., conglutinate) network (J. Lannutti *et al.*, 2006)

Conglutinated networks appear to be useful in some situations where a well established network is desirable. Bead-on-a-string morphologies are also sometimes observed. This is attributed to a complex interaction between solution viscosity, net charge density, and surface tension (Fong *et al.*, 1999).

Deliberate neutralization of the charge on a fluid jet allows the fluid to retract towards droplet form and this causes bead formation. In some cases, as the viscosity is decreased, fiber diameter is decreased and the spindle-shaped beads become more spherical. Higher flow rates are observed to have similar effects. The presence of beads is reportedly reduced by the addition of a secondary solvent that increased conduction through the polymeric solution (Lee *et al.*, 2003).

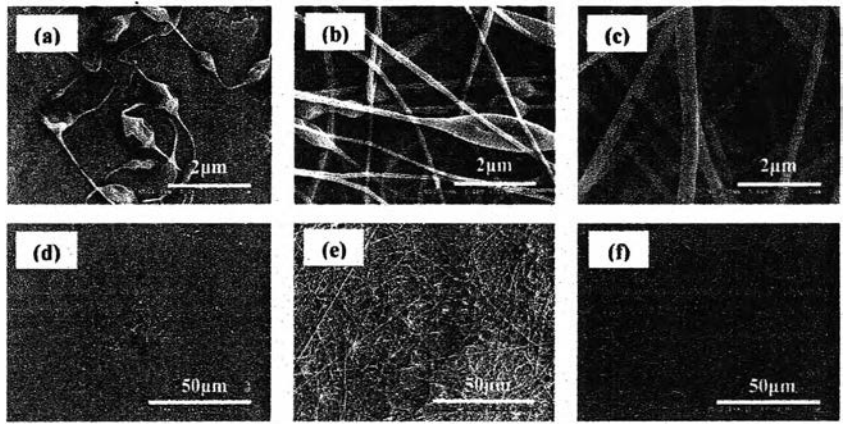

Figure 2.3 SEM images of GE fibers electrospun from gelatin solutions with different concentrations (solvent) mixture of water and ethanol with a water-to-ethanol volume ratio of 9/1): (a,d) 4.5, (b,e) 10, and (c,f) 15% (w/v). Magnification: (a-c) 20 000_, (d-f) 1000_ (Junxing Li *et al.*, 2006).

The possible effects of beads on biological activity such as cellular proliferation, growth and adhesion are not clearly anticipated. On the positive side, understanding of the development of beads provides another parameter for design of scaffolds. For example, beads may prove to be useful as reservoirs of therapeutics. However, the

presence of such beading can lead to practical difficulties associated with cell counting by manual techniques because in either scanning electron or transmission optical microscopy, these beads closely resemble cells. For reasons that are not clear, use of a length of plastic tubing between the solution reservoir and the charged metal needle can reduce beading even though the polymer and solvent combination remains unchanged.

2.5 Degradation

A clear example of technical need for the successful development of scaffolds made from electrospun fibers is how their mechanical behavior is altered during in vitro and in vivo exposures. Increased understanding of the basic principles governing tissue formation requires an improved picture of how the structure and mechanical properties of the underlying scaffold change with time. Whether or not the cell plus scaffold construct will have the appropriate overall mechanical properties in the application (which will vary widely depending upon the target) also depends to an extent upon the degradation of the mechanical properties versus time. Transport and retention of signaling molecules in the vicinity of the fibers is also an unexamined issue.

2.6 Adding bioactive function

While electrospun materials have morphological resemblance to the natural tissues the chemical similarities are presently less than ideal as practical synthetic polymers can typically provide morphological not biochemical biomimesis. There have been efforts to label polymer surfaces with specific chemical signaling factors (Ma *et al.*, 2007) as well as direct electrospinning of polymer plus biomolecule combinations. These efforts seem unlikely to produce a long-term solution but many other synthesis methods are available. If conformationally sensitive biomolecules are present, questions and concerns regarding stability or activity in the resulting electrospun polymer fiber are inevitable (Woerdeman *et al.*, 2005). In addition, the majority of the biomolecule embedded in a fiber may never be exposed at the external surface of a scaffold during critical portions of cellular growth and proliferation. Chemically attached biomolecules (Ma *et al.*, 2007) benefit from the high innate surface area of electrospun materials.

2.7 Materials

Both natural and synthetic materials have been used to produce bioscaffolds depending on the specific types of bioscaffold needed. Some types of bioscaffold need to be biodegradable, for example: scaffolds for nerve repair. Others such as fractured bone repair can use both permanent and biodegradable bioscaffolds. Most of these materials have been known in the medical field long before the topic of tissue engineering came into focus for example, collagen or some linear aliphatic polyesters.

Bioscaffolds may be constructed from natural materials, such as different derivatives of the extracellular matrix which have been studied to evaluate their ability to support cell growth. Proteic materials such as glycosaminoglycans (GAGs), have been validify suitable in terms of cell compatibility, although some issues with potential immunogenicity still remains.

Naturally derived polymers such as collagen, gelatin, chondroitin, and hyaluronae, have been widely used to fabricate scaffolds for cartilage tissue engineering in recent years. hyaluronate was a major component of ECM in cartilage tissue and known to interact with chondrocytes via various surface receptors including CD44, which trigged a sophisticated signaling pathway making chondrocytes retain their original phenotype (Chow *et al.*, 1995). The physicochemical properties of gelatin were suitably modulated due to the existence of many functional groups and without any antigenicity (Takagi, 2004); chondroitin in the scaffold promoted the secretion of proteoglycan and type II collagen (Sechriest *et al.*, 2000).

2.7.1 Hyaluronic acid

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide consisting of alternating disaccharide units of a-1,4-D-glucuronic acid and b-1,3-N-acetyl-D-

glucosamine with a molecular weight range from 103 to 107 (Laurent *et al.*,1995). This polyanionic polymer can be found in connective tissues such as umbilical cord, synovial fluid, vitreous, etc (Laurent,1970). Because of its unique viscoelastic properties and good biocompatibility, unmodified and derivatized HAs have been extensively utilized in the fields of drug delivery, cell encapsulation and tissue regeneration (Saettone *et al., 1994).* Since the hydrophilic, polyanionic surfaces of HA biomaterials do not thermodynamically favor the cell attachment and subsequent tissue formation (Shu *et al.*, 2003), two major issues need to be addressed before HA can be utilized in cell growth and tissue remodeling.

One is to improve the cell attachment onto HA-based biomaterials. Several strategies have been developed including physically coating extracelluar matrix (ECM) proteins such as type I collagen and fibronectin (FN) onto HA biomaterials (Ramamurthi *et al,*,2002), or covalently linking FN functional domains (FNfds) to the HA backbone, which enhances cell interactions with HA (Ghosh *et al.*,2006). The other one is to fabricate an HA microporous scaffold which serves to direct the growth of cells within the scaffold. Solvent casting and lyophilization have been utilized to obtain cross-linked HA hydrogel films as a tissue scaffold material for cell seeding and controlled drug delivery. It was found that cells seeded on solvent casting HA hydrogel films can only attach to the surface and proliferate in a 2D geometry (Luo *et al.*, 2000).

2.7.2 Gelatin

Gelatin is a protein biopolymer derived from partial hydrolysis of native collagens, which are the most abundant structural proteins found in the animal body of skin, tendon, cartilage and bone (Wang *et al.*, 1977). Due to it's biological origin, nonimmunogenicity, biodegradability, biocompatibility, and commercial availability at relatively low cost, gelatin has been widely used in the pharmaceutical and medical fields as sealants for vascular prostheses (Guidoin *et al.*,1987), carrier for drug delivery (Li *et al.*,1998) and dressings for wound healing (Choi *et al.*, 1999).

2.8 Crosslinking Gelatin

Gelatin is the main component of the bioscaffold, but the main problem is that it is water soluble. Therefore fabricated gelatin-based bioscaffolds must go through cross-linking process to make them water-insoluble. Two fundamental methods of cross-linking have been described for gelatin: physical and chemical. Physical methods include UV-irradiation and dehydrothermal treatment, although these are inefficient and make it difficult to control the cross-linking density of the gelatin matrix (Kuijpers *et al.*, 2000).

Chemical cross-linking agents have been categorized into two types: non-zero length and zero-length. Non-zero length cross-linkers are bi-functional or polyfunctional and operate by bridging free carboxylic acid residues or amine groups between adjacent protein molecules. Examples include aldehydes (i.e. formaldehyde, glutaraldehyde, glyceraldehyde), polyepoxides, and isocyanates (Kuijpers *et al.*, 2000). Zero-length cross-linking agents activate carboxylic acid residues to react directly with amine groups on adjacent protein chains. No intervening molecules are introduced between the cross-linked residues, so this process is able to achieve gelatin matrix cross-linking without integrating foreign molecules into the network. Cross-linking agents in this category include acyl azides (Kuijpers *et al.*, 2000) water-soluble carbodiimides (Yabata *et al.*, 1994).

Cross-linking density can be determined by performing a tensile test on gelatin sheets (Iwanaga *et al.*, 2003). Another measure of cross-linking extent for hydrogels is water content, which is defined by the weight percentage of water in a swollen hydrogel. Hydrogel weight before and after swelling in 37 8C in PBS can be measured in order to calculate water content. Control over the cross-linking density of gelatin hydrogel carriers is possible with the use of glutaraldehyde as the cross-linking agent for example (Iwanaga *et al.*, 2003). By either prolonging the cross-linking reaction period or increasing the glutaraldehyde concentrations, the cross-linking density of the gelatin can

be increased, thus allowing for the fabrication of gelatin hydrogel carriers with specific extents of cross-linking by optimization of the cross-linking reaction conditions.

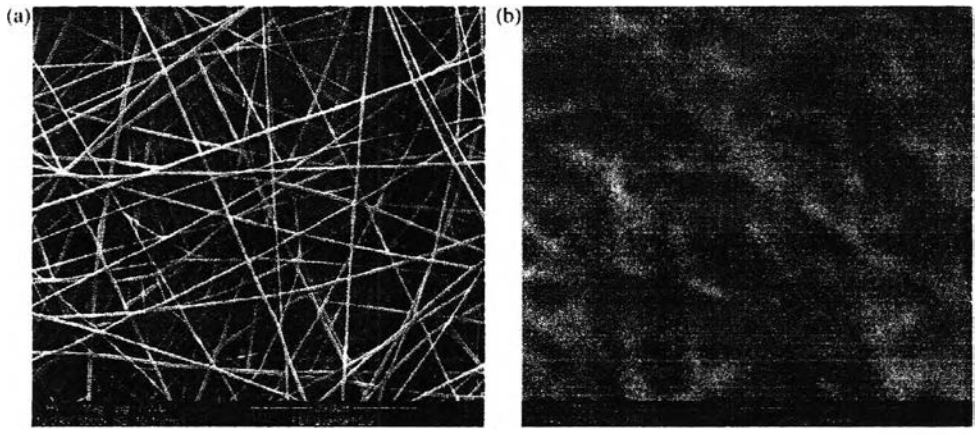

Figure 2.4 Morphologies of gelatin nanofibers: (a) from electrospinning of a 10% w/v gelatin/TFE, and (b) the smeared surface layer of gelatin nanofibrous membrane after adding a drop of water (Y.Z. Zhang *et al.*, 2006).

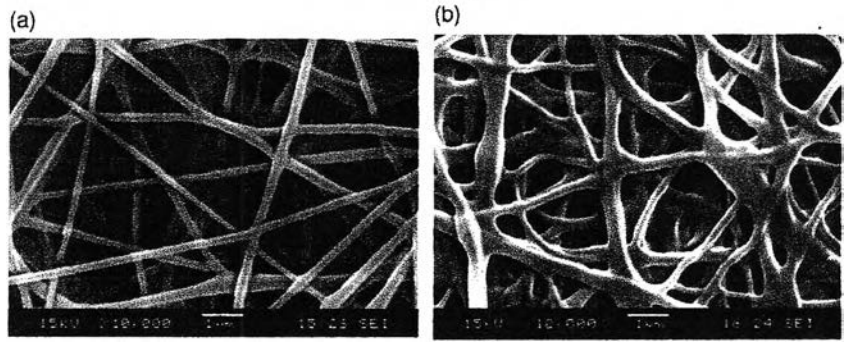

Figure 2.5 SEM micrographs for electrospun collagen–GAG scaffolds: (a) noncrosslinked collagen–GAG scaffold (COLL–GAG) (b) crosslinked collagen– GAG scaffold by GA vapor (X-COLL–GAG) (Shao Ping Zhong *et al.*, 2007).