CHAPTER II
LITERATURE REVIEWS

2.1 Electrospinning

2.1.1 Fundamentals of Electrospinning

Electrospinning or electrostatic spinning is a process for the production of ultrafine polymer filaments using an electrostatic force by introducing a high electrical charge into a polymer solution or melt. The polymer filaments were formed between two electrodes bearing electrical charges of opposite polarities. One of the electrodes was placed into the solution and the other onto a collector. When the polymer solution was ejected out of a metal spinneret with a small hole, the charged solution jets evaporated to become fibers which were collected on the collector.

There are basically three components in electrospinning process: a high voltage supplier, a capillary tube with a needle of a small inner diameter, and a metal collecting screen. The function of a high voltage supplier is used to create an electrically charged jet of polymer solution or melt out of the spinneret. Before reaching the collecting screen, the ejected jet evaporates or solidifies, leaving ultrafine fibers on the collecting screen. One electrode is placed into the spinning solution/melt and the other attached to the grounding collector. The experimental set-up of electrospinning process is shown in Figure 2.1. The electric field is subjected to the end of the capillary tube that contains the solution fluid held by its surface tension. This induces a charge on the surface of the liquid. Mutual charge repulsion and the contraction of the surface charges to the counter electrode cause a force directly opposite to the surface tension (Huang *et al.*, 2003).

As the intensity of the electric field increases, the hemispherical surface of the fluid at the tip of the capillary tube elongates to form a conical shape, known as the Taylor cone (Taylor, 1969). Further increasing the electric field, a critical valve is attained with which the repulsive electrostatic force overcomes the surface tension and the charged jet of the fluid is ejected from the tip of the Taylor cone. The discharged polymer solution jet undergoes an instability and elongation

process, which allows the jet to become very long and thin. Meanwhile, the solvent evaporates, leaving behind a charged polymer fiber. In the case of the melt the discharged jet solidified when it travels in the air.

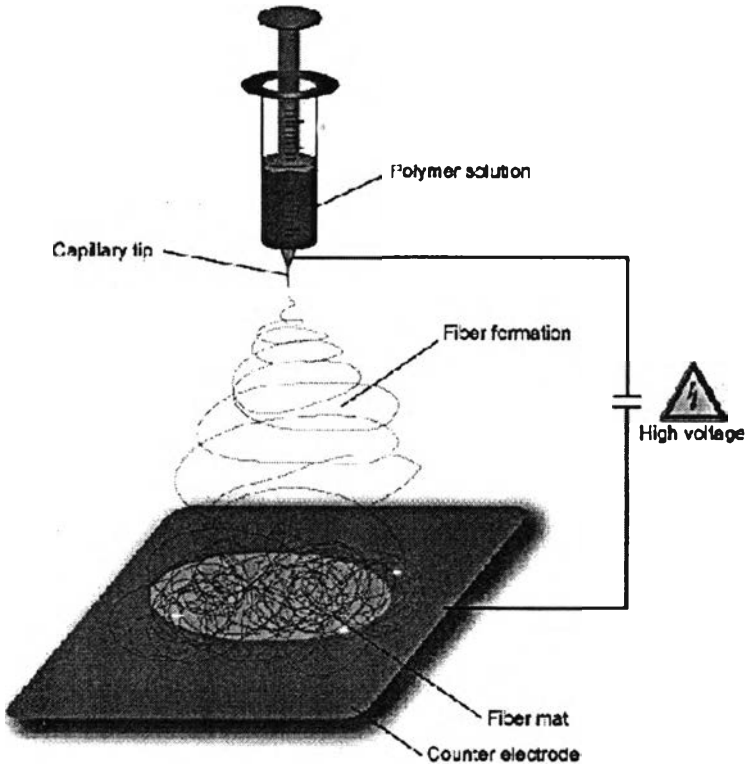

Figure 2.1 Schematic of the electrospinning setup.

In general, the experimental setup can be divided into two types based on direction of polymer supplying nozzle including horizontally and vertically. The two kinds of polymer supplying nozzle are glass pipette and metal needle. For the experimental setup where glass pipette is used, the external potential was supplied by directly inserting a metal electrode to the capillary tube filled with a polymer solution (Reneker *et al.*, 1996).

Most nanofibers obtained so far are in non-woven form, which can be useful for applications such as filtration, tissue scaffolds, implant coating film, and wound dressing. There have been efforts of fabricating continuous, single fiber strand using this technique. A simple technique suggested the use of a rotating device with angular velocity of up to thousands of rounds per minute (rpm). Researchers

from Virginia Commonwealth University (Boland et al., 2001) used this technique to obtain aligned electrospun poly(glycolic acid) (PGA) (at 1000 rpm) and type I collagen (at 4500 rpm rotating speed) fibers.

Many parameters can influence the transformation of polymer solutions into nanofibers through electrospinning. These parameters include (a) the solution properties such as viscosity, elasticity, conductivity, and surface tension, (b) governing variables such as hydrostatic pressure in the capillary tube, electric potential at the capillary tip, and the gap (distance between the tip and the collecting screen), and (c) ambient parameters such as solution temperature, humidity, and air velocity in the electrospinning chamber (Huang et al., 2003)

One of the most important quantities related to electrospinning is the fiber diameter. Since nanofibers are resulted from evaporation or solidification of polymer fluid jets, the fiber diameters will depend primarily on the jet sizes as well as on the polymer contents in the jets. It has been recognized that during the traveling of a solution jet from the pipette onto the metal collector, the primary jet may (Bergshoef and Vancso, 1999) or may not (Reneker et al.,2000) be split into multiple jets, resulting in different fiber diameters. Another parameter influencing the fiber diameter is the solution viscosity. A higher viscosity results in a larger fiber diameter (Doshi and Reneker, 1995). However, when a solid polymer is dissolved in a solvent, the solution viscosity is proportional to the polymer concentration. Thus, the higher the polymer concentration the larger the resulting nanofibers diameters will be. Another parameter affecting the fiber diameters to a remarkable extent is the applied electrical voltage. In general, a higher applied voltage ejects more fluid in a jet, resulting in a larger fiber diameter (Demir MM., 2002).

2.1.2 Polymer Types

More than 50 different polymers have been successfully electrospun into ultrafine fibers with diameters ranging from less than 3 nm to over 1 μm. Most of the polymers were completely dissolved in solvents before electrospinning. Both the dissolution and the electrospinning should be carried out at ambient condition. However, some polymers may emit unpleasant or even harmful smells so the processes should be carried out in a hood with a good ventilation.

Molten polymers can also be made into nanofibers through electrospinning. Instead of a solution, a polymer melt is introduced into the capillary tube; the electrospinning process for a polymer melt has to be performed in a vacuum condition, for example, polyethylene (PE) (Larrondo *et al.*, 1981), polypropylene (PP) (Broda *et al.*, 2003). However, fewer polymers have been electrospun in their molten state.

2.1.3 Fibers Characterization

Geometric properties of nanofibers such as size and its distribution, orientation, and morphology (e.g. cross-sectional shape and surface roughness) can be characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) (Li *et al.*, 2002). The use of TEM does not require the sample in a dry state as that of SEM. Hence, nanofibers electrospun from a polymer solution can be directly observed under TEM.

Chemical integrity of nanofibers can be characterized by Fourier-transformed infrared spectroscopy (FTIR) (Grählert *et al.*, 1999) and nuclear magnetic resonance (NMR) techniques (Bourbigot *et al.*, 2000). Supermolecular structure describes the configuration of the macromolecules in the fibers, which can be characterized by optical birefringence (Buchko *et al.*, 1999), wide-angle X-ray diffraction (WAXD), small-angle X-ray scattering (SAXS) and differential scanning calorimetry (DSC) (Zussman *et al.*, 2002).

The conductivity characterizing the electrical transport properties of the electrospun nonwoven ultra-fine mats of polyaniline doped with camphorsulfonic acid blended with PEO (polyethylene oxide) was studied (Norris *et al.*, 2000). Since the non-woven mat was highly porous and the fill factor of the fibers was less than that of a cast film, the measured conductivity seemed to be lower than that of the bulk.

2.2 Peripheral Nerve Regeneration

2.2.1 Nerve System

The nervous system can be divided into two major divisions: the central nervous system (CNS) and the peripheral nervous system (PNS). The central

nervous system (CNS) includes the brain and spinal cord that consists of a vast number of neurons, astroglia, microglia and oligodendrocytes that act to coordinate, recognize, initiate, propagate and process signals from external and internal stimuli whereas the peripheral nervous system (PNS) contains only ganglia, motor neurons, sensory nerves and the bundles of neurons for connecting the CNS to other parts of the body. (Seal et. Al., 2001)

The functional unit of the nervous system is the neuron. In humans, there are about 100 billion neurons with varying size and shape in their brain alone. Although many different types of neurons exist, the general morphology of neurons usually includes a cell body or soma, dendrites and axon as shown in Figure 2.4. Dendrites are thread-like branches that receive information from another cell and transmit the message to the cell body and conduct away to the target through the axon. The nervous system can monitor and control almost every organ system through a series of positive and negative feedback loops. By means of ionic currents, neuron act to conduct electrical impulses from the dendrites, which serve as signal receivers, through the soma onto the axon terminal, the signal transmitter. Once the signal reaches the end of axon, several vesicles containing chemicals called neurotransmitters release their contents into the region between the axon terminus and its target (another neuron or muscle fiber) called the synaptic cleft. In the case of motor neuron, for examples, this electrical causes muscle activation through the release of acetylcholine.

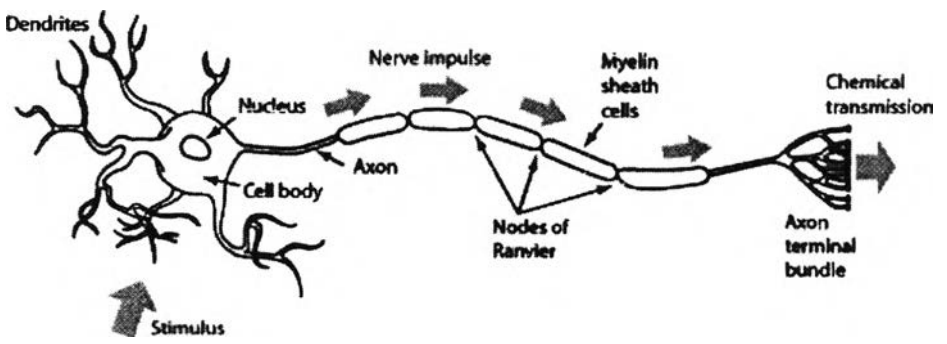

Figure 2.2 The structure of a PNS neuron.

In PNS neuron, some axons are wrapped in a myelin sheath formed from the plasma membranes of specialized glial cells known as Schwann cells. These glial cells serve as supportive, nutritive, service facilities for neurons and also aid in accelerating the propagation velocity of electrical impulses by insulating the neuron with a myelin sheath. In nerve reparing, Schwann cells also aid in cleaning up PNS debris and guide the regrowth by producing growth factors and the excretion of extracellular matrix. To do this, Schwann cells arrange themselves in a series of cylinders that serves as a guide for sprouts of regenerating axons. When one of these sprouts encounters a cylinder, the sprout will grow through these tubes.

2.2.2 Wallerian Degeneration Process

Injury to the PNS initiates a sequence of striking and characteristic changes in the distal nerve stump, which are collectively referred to as *Wallerian degeneration*. The process of Wallerian degeneration is summarized as follows. (Dezawa and Adachi-Usami, 1999)

1. At first, axons degenerate, and the myelin sheaths break up into ovoids.

2. The myelin debris is then phagocytosed by macrophages that invade the degenerating nerve.

3. The basement membrane that surrounds each Schwann cell remains intact, and Schwann cells remain within their basement membrane. Those Schwann cells in the distal nerve stump become mitotic and proliferate. This mitotic activity results in the formation of cordons of Schwann cells, referred to as band of Büngner. These dividing Schwann cells are expressing connexin and are coupled through gap junctional channel. Accordingly, Schwann cells and their associated basement membrane persist, while axons and myelin sheaths degenerate during Wallerian degeneration.

4. Proximal to the injury site, axons give rise to one or more growth cones. The growth cones enter into the Schwann cell tubes, namely bands of Büngner, which form the pathway for regenerating axons to the targets. Initially, one Schwann cell surrounds several regenerating axons, but eventually they segregate to make a 1:1 relationship between the axon and Schwann cell. A new basement membrane forms around the outer surface of the Schwann cell, and a new myelin

sheath is wrapped around the regenerated axon.

In the time course of Wallerian degeneration and subsequent regeneration in PNS, Schwann cells produce various kinds of factors and cytokines, and cell adhesion molecules. PNS regeneration may be induced and supported by the integrated functions of these factors.

2.2.3 The Role of Schwann Cells in Nerve Regeneration

Schwann cells play a crucial role in nerve regeneration through the production of growth factors, extracellular matrix and cell adhesion molecules. The functional substances produced by Schwann cells are summarized in Figure 2.5.

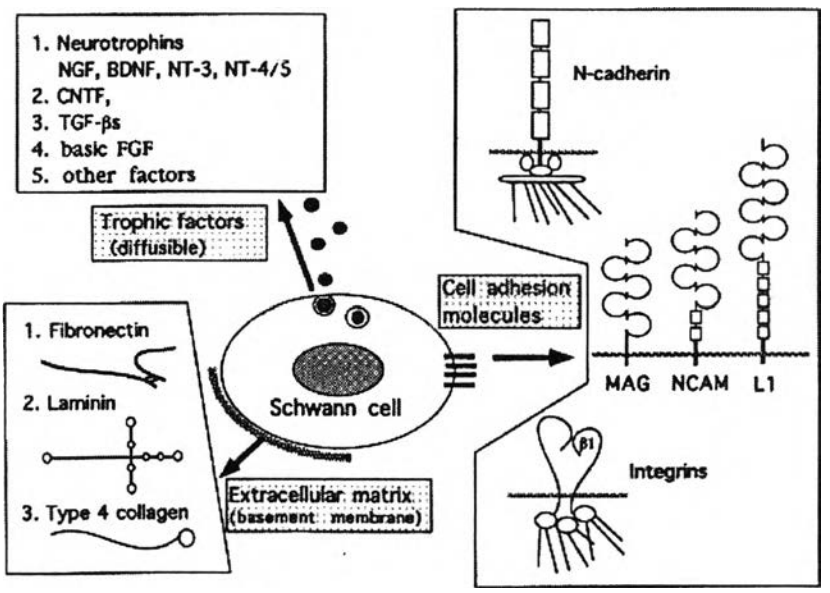

Figure 2.3 Summary of functional substances produced by activated Schwann cells.

2.2.3.1 Trophic Factors

It is known that Schwann cells produce many kinds of neurotrophic factors. The factors related with nerve regeneration belong to the neurotrophin family. Four members of neurotrophin family are relevant in mammalian nervous system; NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). NGF is a well characterized neurotrophin which may directly influence on axonal regeneration. After transection of a peripheral nerve, NGF sharply increases in Schwann cells and in the fibroblasts of distal segment. Increased

synthesis of NGF in Schwann cells appears to enhance the survival and sprouting of nerve cells. In addition, NGF was reported to increase the expression of cell adhesion molecule L1 in Schwann cells, which promotes the migration of Schwann cells. L1 is one of the mediators of RGC neurite outgrowth on Schwann cells and NGF has been proposed to enhance axonal regeneration partly by stimulating production of L1 by Schwann cells. BDNF and NT-4/5 also increase after peripheral nerve injury, but more slowly than the case of NGF. In contrast, the level of NT-3 mRNA decreases after nerve injury. Taken together, NGF, BDNF and NT-4/5 are synthesized by Schwann cell during Wallerian degeneration and may promote the survival and axonal sprouts of regenerating neuronal cells.

2.2.3.2 Extracellular Matrix

Schwann cells are known to express a variety of extracellular matrix including fibronectin, laminin, tenascin-C, type IV and VI collagen, heparan sulfate, chondroitin sulfate proteoglycans and entactin/nidogen that act as substrate for nerve regeneration. Among these, fibronectin, tenascin-C, laminin and merosin (laminin-2) are the most prominent extracellular matrix glycoproteins that are involved in neural regeneration. As to tenascin-C, it sometimes promotes neurite outgrowth, while often inhibits the regeneration.

Several studies showed that basement membrane of Schwann cells provides good substrate for regenerating axons. After repetitive freezing and thawing to kill the Schwann cells, growth cones are still able to adhere to the inner surface of the basement membrane. Therefore, at least in the peripheral nerve, the basement membrane is considered to be a substrate capable of supporting axonal regeneration. However, most growth cones appear to prefer Schwann cells when they are given a choice of either denervated Schwann cell or basement membrane (Martini, 1994).

2.2.4.3 Cell Adhesion Molecules

Many of the glycoproteins involved in the cell adhesion belong to one of the three major structural families. The first is the immunoglobulin superfamily, which includes some of the best characterized cell adhesion molecules in the nervous system, such as NCAM, L1 and MAG. The second family comprises a group of structurally related glycoproteins called cadherins, of which N-cadherin is a

prominent member expressed within the nervous system. The third family consists of a large family of glycoproteins called integrins. Integrins consist of two noncovalently linked subunits of α and β, and mediate interactions between the cell surface and molecules in the extracellular matrix. Different sets of α and β subunits are expressed by distinct cell types. The particular combination of subunits defines which set of extracellular and matrix proteins will be recognized by each integrin. Immunoglobulins and cadherins mediates the binding of cells by a homophilic mechanism, that is, a molecule on one cell binds to the same molecule on an adjacent cell, although in some cases immunoglobulins bind to other members of this superfamily. Cell adhesion molecules are important neuronal receptors mediating substrate-induced axonal growth. Recently it is suggested that signaling of axon growth through these receptors involves both regulation of tyrosin phosphorylation and transient increases in intracellular Ca^{2+}, possibly links to cytoskeletal rearrangements and directed addition of plasma membrane.

Denervated Schwann cells express a variety of cell adhesion molecules including NCAM, L1 and N-cadherin. The role of these molecules in promoting axonal outgrowth has been investigated by co-culturing neurons and Schwann cells in the presence of blocking antibodies to each molecule. The results showed that all of these cell adhesion molecules play some role in promoting regeneration by interacting with their counterparts on growth cones. In particular, antibodies to L1 or L1-like antigen abolished RGC neurite outgrowth on Schwann cell in rat (Kleitman *et al.*, 1988), and on oligodendrocyte in goldfish (Ankerhold *et al.*, 1998), but only partially inhibits outgrowth of PNS neurons. Recent analysis using knockout mice showed that the absence of NCAM strongly affects fasciculation, pathfinding and plasticity of axons, and that the disruption of L1 resulted in defects in the development of neuronal tracts and impairment of learning. As to integrins, the β1 integrins seem to be important, since antibodies to this subunit inhibited the neuronal outgrowth on extracellular matrix proteins (Agius and Cochard, 1998). Furthermore enhanced expression of α5β1 integrin and its ligand fibronectin has been shown to occur in regenerating peripheral nerve (Lefcort *et al.*, 1992). Taken together, this suggests that growth cones principally use NCAM, L1

and N-cadherin to extend on Schwann cells, and β1 integrins to grow on Schwann cell extracellular matrix.

2.2.4 Nerve Regeneration

Every year, a lot of people are disabled by neurological disease and injury, resulting in the loss of functioning neuronal circuits in either peripheral nervous system (PNS) or central nervous system (CNS). When the nerve system loses their function, it has little capacity for self-repairing and neurons are not capable of proliferating in their native environment. The standard therapies used for the restoration of neuronal function are the autograft technique by taking nerve tissue from a donor site and grafted into the injured site. However, this method is frequently associated with donor site morbidity and incomplete functional recovery. One possible alternative to repair nerve defects involves the use of artificial nerve materials that create a favorable environment for nerve regeneration.

The artificial nerve graft technique involves the introduction of both ends of injured nerve stumps into a tubular chamber which aid guidance of growing fibers along appropriate paths by mechanical orientation and confinement, enhancing the precision of stump approximation and minimizing invasion and scarring of the nerve. Several synthetic materials, either nondegradable and or biodegradable, have been used as a nerve conduit. Due to the conduit must implantation in human body so the major problem for using nondegradable conduits is that they remain in situ as foreign bodies after the nerve has regenerated and need second surgery to remove the conduits, causing possible to damage the nerve. Therefore, biodegradable and biocompatible conduits seem a more important material to reconstruct nerve gaps. However, biodegradable conduits are degraded as a function of time, thus may lose their functional capability as a structural degradation. So, an ideal nerve guide should possess the following characteristics including inertness, biocompatibility and bioresorbility, thinness, flexibility and has appropriate mechanical properties and being capability to inhibit pathological processes and beneficial to healing and regeneration.

Despite these advances and contributions in the field of tissue engineering, current results with nerve conduits have failed to equate with nerve regeneration equivalent to autogenous grafts for large distances. The failure is

proposed to be the result of combination factors, such as the inability of neurotrophic factors produced by distal stumps to reach the proximal stump, the lack of a matrix bridge as a supporting structure, and the absence of Schwann Cells (SCs) that act as supportive, nutritive and service facilities for neurons regrowth. Nevertheless, an ideal nerve guidance material remains a challenge.

A variety of biocompatible materials have been investigated for their suitability in tissue engineering application. Several techniques have been developed to process synthetic and natural scaffold materials, which precisely mimic a natural repairing process in the human body. Nerve guide channel produced by using phase-segregated of poly[(R)-3-hydroxybutyric acid-co-(R)-3-hydroxyvaleric acid]-diol (PHB) and poly[glycolide-co-(e-caprolactone)]-diol multiblock copolymer coupled with aliphatic, 2,2,4 trimethylhexamethylene diisocyanate (TMDI) as chain extender (Borkenhagen M. et al.,1998). Tubular structures were fabricated from three different types of materials containing 41, 17 or 8 wt% PHB. Nerve regeneration through a 10 mm long NGC using a transected sciatic nerve model with an 8 mm gap was studied in rats at 4, 12 and 24 weeks. Nerve regeneration results show the regenerated tissue cables centrally within the channel lumen and composed of numerous myelinated axons and Schwann cells and no significant difference in the degree of regeneration was observed between the various channel types.

Chen et al. (2005) evaluated peripheral nerve regeneration conduit from genipin-cross-linked gelatin by using dip coating technique. After subcutaneous implantation on the dorsal side of the rat, the GGC only evoked a mild tissue response, forming a thin tissue capsule surrounding the conduit. The results from biodegradability and its effectiveness as a guidance channel showed that the tube fragmentation was not obvious until 6 weeks post-implantation and successful regeneration through the gap occurred in all the conduits at the three experimental periods of 4, 6, and 8 weeks. Histological observation showed that numerous regenerated nerve fibers, mostly unmyelinated and surrounded by Schwann cells.

By enhancing cell adhesion, viability and neurite extension, Bender et al. (2004) created the new methods to fabricate porous, biodegradable conduits composite nerve guides comprised of poly(caprolactone) (PCL) and porous collagen-based beads (CultiSphers). Yua and Shoichet (2005) prepared the peptide modified

scaffolds copolymerization of 2-hydroxyethyl methacrylate with 2-aminoethyl methacrylate, P(HEMA-co-AEMA) by using laminin-derived peptides for enhancing cell adhesion and neurite outgrowth. The synthesized scaffolds have numerous longitudinal oriented channel with a compressive modulus which to match the modulus of soft nerve tissue and enhanced neural cell adhesion and guided neurite outgrowth than non-peptide modified controls

The electrospun poly(L-lactide-*co*-glycolide) biodegradable scaffold was investigated the potential to use as nerve guidance channels (Bini *et al.*, 2004). The nanofibrous scaffold was flexible, permeable and showed no swelling. After implantation of the nanofibres scaffold in the rat sciatic nerve model, there was no inflammatory response and showed successful nerve regeneration. Yang et al. (2005) studied the efficiency of aligned poly(L-lactic acid) (PLLA) nano and micro fibrous scaffolds on neural tissue outgrowth and compare their performance with random PLLA scaffolds by using electrospinning technique. The results of cell culture after 1 day show that the direction of NSC elongation and its neurite outgrowth is parallel to the direction of PLLA fibers for ANF and AMF scaffolds, whereas it was random for RNF and no significant changes were observed on the cell orientation with respect to the fiber diameters. However, the rate of NSC differentiation was higher for PLLA nanofibers than that of micro fibers and it was independent of the fiber alignment.

The potential to use chitin and chitosan for biodegradable nerve guides was investigated by using acylation chemistry and mold casting techniques (Freier et al., 2005). Chitosan films showed significantly enhanced neurite outgrowth relative to chitin films, reflecting the dependence of nerve cell affinity on the amine content in the polysaccharide. This implies that cell adhesion and neurite extension can be adjusted by amine content, which is important for tissue engineering in the nervous system. The interaction between Schwann cell and chitosan was evaluated by immunocytochemical staining and measuring viability of Schwann cells. The experimental results indicated that Schwann cells could grow onto chitosan materials with two different shapes: spherical and long olivary and there was a good biological compatibility between chitosan and Schwann cells.

2.3 Chitin and Chitosan

Chitin, which is the most abundant biopolymer in nature after cellulose, is a polysaccharide widely distributed in nature as the principal component of exoskeletons of crustaceans and insects as well as of cell walls of some bacteria and fungi. Like cellulose, it is a glucose-based unbranched polysaccharide. It differs from cellulose at the C-2 carbon by having an acetamido residue in place of a hydroxyl group. Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after alkaline deacetylation of the chitin. It comprises copolymers of glucosamine and N-acetyl glucosamine and the term chitosan embraces a series of polymers which vary in molecular weight (from about 10,000 to 2 million Dalton). Chitosan is semi-crystalline and the degree of crystallinity is a function of the degree of deacetylation. Crystallinity is maximal for both chitin (i.e. 0% deacetylated) and fully deacetylated (i.e. 100%) chitosan. Minimal crystallinity is achieved at intermediate degrees of deacetylation. Currently, chitin and chitosan are manufactured commercially in large scale from the outer shell of shrimps, lobsters and crabs.

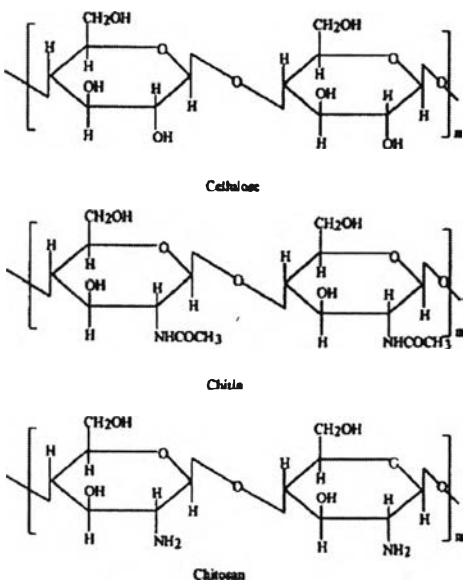

Figure 2.4 Structures of cellulose, chitin and chitosan.

2.3.1 Processing of Chitin and Chitosan

Chitin is easily obtained from crab or shrimp shells and fungal *mycelia*. In the first case, chitin production is associated with food industries such as shrimp canning. In the second case, the production of chitosan–glucan complexes is associated with fermentation processes, similar to those for the production of citric acid from *Aspergillus niger*, *Mucor rouxii*, and *Streptomyces*, which involves alkali treatment yielding chitosan–glucan complexes. The alkali removes the protein and deacetylates chitin simultaneously. Depending on the alkali concentration, some soluble glycans are removed. The processing of crustacean shells mainly involves the removal of proteins and the dissolution of calcium carbonate which is present in crab shells in high concentrations. The resulting chitin is deacetylated in 40% sodium hydroxide at 120°C for 1–3 h. This treatment produces 70% deacetylated chitosan as in Figure 2.7.

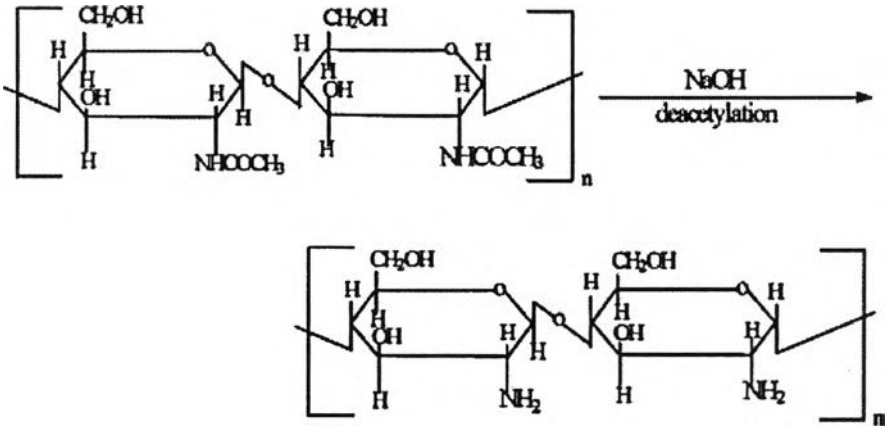

Figure 2.5 The structure of deacetylated chitosan.

2.3.2 Properties of Chitin and Chitosan

Most of the naturally occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans, are neutral or acidic in nature, whereas chitin and chitosan are examples of highly basic polysaccharides. Their unique properties include polyoxysalt formation, ability to form films, chelate metal ions and optical structural characteristics. Like cellulose, chitin functions

naturally as a structural polysaccharide, but differs from cellulose in its properties. Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugation with aqueous solutions of mineral acids and dimethylacetamide containing 5% lithium chloride. Chitosan, the deacetylated product of chitin, is soluble in dilute acids such as acetic acid, formic acid, etc.

Chitosan, which is polycationic in acidic environments, possesses an ability to form gels at acidic pH values because it is hydrophilic and can retain water in its structure. The acetylation of chitosan in hydroalcoholic media allows the selective modification of the free amino groups and is responsible for a process of gelation. It has been shown that the charge density of the chain segments is an essential parameter for the formation of gels and all factors that lower this parameter favor deswelling and reversibility. The high hydration, the physicochemical and physical properties, as well as the polyelectrolyte behavior of this kind of gel allow applications such as bioactive dressing for wound healing. Gels can also be used as a slow-release drug-delivery system. The solubility of chitosan can be sharply reduced by cross-linking the macromolecules with covalent bonds using, for examples, glutaraldehyde. Swelling of the films decreases with an increase in the amount of cross-linking agent added. The swelling of enzyme-containing films decreases more significantly, probably because of formation of additional crosslinks due to the participation of the functional groups of the enzyme in the reaction.

Recently, the gel forming ability of chitosan in *N*-methylmorpholine *N*-oxide and its application in controlled drug release formulations has been reported (Dutta et al, 1997). The hydrolysis of chitin with concentrated acids under drastic conditions produces relatively pure D-glucosamine. The nitrogen content of chitin varies from 5 to 8% depending on the extent of deacetylation, whereas the nitrogen in chitosan is mostly in the form of primary aliphatic amino groups. Chitosan, therefore, undergoes reactions typical of amines, of which *N*-acylation and Schiff reaction are the most important. Chitosan derivatives are easily obtained under mild conditions and can be considered as substituted glucans. *N*-Acylation with acid anhydrides or acryl halides introduces amino groups at the chitosan nitrogen. Acetic anhydride affords fully acetylated chitins. Linear aliphatic *N*-acryl groups above

propionyl permit rapid acetylation of hydroxyl groups. Higher benzoylated chitin is soluble in benzyl alcohol, dimethylsulfoxide, formic acid and dichloroacetic acid. The *N*-hexanoyl, *N*-decanoyl and *N*-dodecanoyl derivatives have been obtained in methanesulfonic acid (Nishi et. al, 1979).

At room temperature, chitosan forms aldimines and ketimines with aldehydes and ketones, respectively. Reaction with ketoacids followed by reaction with sodium borohydride produces glucans carrying proteic and non-proteic amino groups. *N*-Carboxymethyl chitosan is obtained from glyoxylic acid. Examples of non-proteic amine acid glucans derived from chitosan are the *N*-carboxybenzyl chitosans obtained from *o*- and *p*-phthalaldehydic acids. Chitosan and simple aldehydes produce *N*-alkyl chitosan upon hydrogenation. The presence of the more or less bulky substituent weakens the hydrogen bonds of chitosan; therefore *N*-alkyl chitosans swell in water in spite of the hydrophobicity of the alkyl chains, but they retain the film forming property of chitosan.

2.3.3 Physical and Chemical Characterization

2.3.3.1 Degree of N-acetylation

An important parameter to examine closely is the degree of *N*-acetylation in chitin. This parameter has a striking effect on chitin solubility and solution properties. Chitosan is the universally accepted non-toxic *N*-deacetylated derivative of chitin, where chitin is *N*-deacetylated to such an extent that it becomes soluble in dilute aqueous acetic and formic acids. In chitin, the acetylated units prevail (degree of acetylation typically 0.90). Chitosan is the fully or partially *N*-deacetylated derivative of chitin with a typical degree of acetylation of less than 0.35. To define this ratio, attempts have been made with many analytical tools, which include IR spectroscopy, pyrolysis gas chromatography, gel permeation chromatography and UV spectrophotometry, first derivative of UV spectrophotometry, ^{1}H-NMR spectroscopy, ^{13}C solid state NMR, thermal analysis, various titration schemes, acid hydrolysis and HPLC, separation spectrometry methods and, more recently, near-infrared spectroscopy.

2.3.3.2 Molecular Weight

Chitosan molecular weight distributions have been obtained using HPLC. The weight-average molecular weight (M_w) of chitin and chitosan has

been determined by light scattering. Viscometry is a simple and rapid method for the determination of molecular weight; the constants α and K in the Mark–Houwink equation have been determined in 0.1 M acetic acid and 0.2 M sodium chloride solution. The intrinsic viscosity is expressed as;

$$[\eta] = KM^{\alpha} = 1.81\times10^{-3}M^{0.93}$$

The charged nature of chitosan in acid solvents and chitosan's propensity to form aggregation complexes require care when applying these constants. Furthermore, converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution, which in turn influences the agglomeration. The weight-average molecular weight of chitin is 1.03×10^{6} to 2.5×10^{6}, but the *N*-deacetylation reaction reduces this to 1×10^{5} to 5×10^{5} (Lee V.F. et. al., 1974).

2.3.3.3 Solvent and Solution Properties

Both cellulose and chitin are highly crystalline, intractable materials and only a limited number of solvents are known which are applicable as reaction solvents. Chitin and chitosan degrade before melting, which is typical for polysaccharides with extensive hydrogen bonding. This makes it necessary to dissolve chitin and chitosan in an appropriate solvent system to impart functionality. For each solvent system, polymer concentration, pH, counterion concentration and temperature effects on the solution viscosity must be known. Comparative data from solvent to solvent are not available. As a general rule, the maximum amount of polymer is dissolved in a given solvent towards a homogeneous solution. Subsequently, the polymer is regenerated in the required form. A coagulant is required for polymer regeneration or solidification. The nature of the coagulant is also highly dependent on the solvent and solution properties as well as the polymer used.