CHITOSAN- POLYCAPROLACTONE MIXTURES AS BIOMATERIALS - INFLUENCE OF SURFACE MORPHOLOGY ON CELLULAR ACTIVITY

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CHITOSAN- POLYCAPROLACTONE MIXTURES AS BIOMATERIALS - INFLUENCE OF SURFACE MORPHOLOGY ON CELLULAR ACTIVITY

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LIST OF ABBREVIATIONS

ΔH: change of enthalpy

2D: two dimensional

3D: three dimensional

AFM: atomic force microscopy

CAM: chorio-allantoic membrane

DD: degree of deacetylation

DMTA: dynamic mechanical and thermal analysis

DSC: differential scanning calorimetry

ECM: extracellular matrix

EFM: electron force microscopy

FTIR: fourier transform infrared spectroscopy

GAGs: glycosaminoglycans

MEF: mouse embryonic fibroblasts

MW: molecular weight

PCL: poly(caprolactone)

PEG: poly(ethylene glycol)

PGA: poly(glycolic acid)

PLA: poly(lactic acid)

PLGA: poly(lactic-co-glycolic acid)

PVOH: poly(vinyl alcohol)

RGD: arginine- glycine- aspartate

SEM: scanning electron microscopy

SIS: sub-intestinal submucosa

T_d: thermal decomposition temperature

T_e: water evaporation temperature

T_g: glass transition temperature

T_m: melting temperature

WAXD: wide angle x-ray diffraction

CHAPTER I

INTRODUCTION

1.1. TISSUE ENGINEERING: Every year, thousands of people succumb to diseased or damaged organs. Until recently, the only solution to this has been organ transplantation from other individuals (allografts) which is severely limited by dearth of organ donors. As of July 6, 2006, in the United States alone, there are 92,322 patients on the waiting list for different organs while only 4785 potential donors are available (United Network for Organ Sharing). Allografts also pose the risk of immunological reaction, requiring life-long immunosuppression which in turn has complicated side effects (Shieh and Vacanti 2005). Other options such as autografts (tissues from the same patient) and xenografts (tissues from other species) have their own limitations. While there may not be sufficient healthy tissue available in the patient for an autograft, xenografts carry the risks of severe immunological reaction and disease transmission. Prosthetic devices pose the problem of infection as they are made of artificial materials such as stainless steel, which are implanted permanently causing inconvenience to the patient.

Tissue engineering has the potential to solve this problem by using cells and biocompatible materials to generate and transplant new and functional tissues and organs. This technology is based on the principle that living cells can be harvested and grown on an appropriate matrix (also termed as scaffold) and stimulated to form specific tissues that mimic the complex structures and physiological behavior of natural tissues while the scaffold degrades away into nontoxic substances in the body (Langer and Vacanti 1993). Cells attach onto the scaffold, then proliferate and colonize under a number of external dynamic and chemical stimuli such as shear stress and growth factors. The extracellular matrix (ECM) organizes into a functional tissue that integrates with the host tissue.

Current limitations of tissue engineering include lack of suitable scaffolds and incomplete understanding of cell-material interactions, especially under dynamic three-dimensional (3D) conditions. The drawbacks of scaffolds in turn arise primarily from shortcomings of the biomaterials used for their fabrication. Much of the success of scaffolds depends on finding an appropriate biomaterial to address the critical physical, mass transport and biological design variables inherent to each application. Recent studies have shown that scaffold morphology such as pore size, shape, and spatial architecture also affect cellular attachment, morphology, and colonization dynamics (O'Brien, Harley et al. 2005; Huang, Siewe et al. 2006).

1.2. NATURAL AND SYNTHETIC BIOMATERIALS: Biomaterials are used not only in tissue engineering scaffolds but also in making medical devices (e.g. sutures and catheters) and drug delivery devices as they are biocompatible i.e. non-toxic and immunogenic materials. For tissue engineering, additionally these materials have to be biodegradable i.e. they must degrade into nontoxic products under physiological conditions, at a matching rate of tissue formation. The biomaterials also need to have adequate mechanical strength for the scaffold to withstand stress and support tissue growth. Importantly, they must have biofunctional (also termed as bioregulatory) properties i.e. support and regulate adhesion, spreading and other cellular mechanisms involved in tissue formation. Based on the source, there are two types of matrices in tissue engineering, viz. natural and synthesized. Natural matrices are acellularized biological tissues such as sub-intestinal sub mucosa (SIS) obtained primarily from xenogeneic tissues (Raghavan, Kropp et al. 2005). SIS is rich in type 1 collagen and unlike other acellular matrices, contains a few growth factors that are useful in tissue regeneration. These matrices offer the advantage of decreased antigenicity and biocompatibility, but they are limited in reliable and reproducible supply and also pose the general threats of xenografts, as discussed before.

Synthesized matrices are non-biological matrices. Prominent among these are polymers, which are used for regeneration of soft tissue such as nerve and skin; whereas ceramics are used for hard tissues such as bone and tooth (Ramakrishna, Mayer et al. 2001). Polymers can again be grouped into two categories, natural and synthetic. Natural polymers such as collagen, gelatin, alginate, chitosan and glycosaminoglycans are harvested from natural sources and hence possess good biocompatibility and bioregulatory properties. However, their mechanical properties are severely restricted (Griffith 2000; Seal, Otero et al. 2001) and it is not easy to alter their physicochemical properties like degradation rate and mechanical strength. Among these natural polymers, chitosan has gained more attention in biomedical applications due to a) the possibility of large scale production and low cost, b) positive charge and reactive functional groups that help regulate cellular activity, and c) antibacterial properties. Chitosan is a derivative of chitin, which is the second most abundant polysaccharide obtained from nature. Chitosan is hemocompatible and non-immunogenic, and degradable into non-toxic oligosaccharides inside the body by the action of lysozymes. Its positive charge and reactive functional groups help complex with other uncharged or anionic polymers and also bind with negatively charged proteins. The physicochemical and mechanical properties of chitosan can be tailored to an extent by altering its molecular weight and degree of deacetylation. Nevertheless, it lacks the tensile strength required to match that of several natural tissues including ligament (29.5MPa), tendon (46.5MPa) and articular cartilage (27.5MPa) (Black and Hastings 1998).

On the other hand, many synthetic polymers exhibit the physicochemical and mechanical properties comparable to those of the biological tissue that they are required to substitute, but are not sufficiently bioactive (Seal, Otero et al. 2001). Polyesters such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL) can be reproduced with specific molecular weights, block structures, degradable linkages and crosslinking modes, and have excellent mechanical strength. Among the synthetic polymers, PCL is one of the most flexible and easiest to process (Wiseman, Domb et al. 1998). It is an FDA approved biomaterial, currently used in drug delivery and sutures (Wise 1995). Its low melting point (60°C) allows easy processing and it is biodegradable by hydrolysis. PCL films have tensile strength upto 31MPa and can elongate upto 940% before break (Ng, Teoh et al. 2000). However, the biofunctional properties of PCL are limited and it degrades very slowly (Wise 1995). In summary, there is a lack of biomaterials that are mechanically strong as well as bioregulatory for tissue engineering. Developing biomaterials with programmable mechanical and biological properties will significantly help tissue regeneration technologies.

1.3. HYPOTHESIS: It is a common practice to combine two or more polymers by blending, grafting or copolymerization to obtain novel polymers (Ramakrishna, Mayer et al. 2001). The individual deficiencies of natural and synthetic polymers can be overcome by combining them to obtain a composite that has all the desirable qualities like high bioactivity, mechanical strength, and tailorable biodegradability. Moreover, biological tissues maybe considered as composite materials with anisotropic properties which are dictated by the spatial arrangement of various tissue components such as collagen, fibrin, and elastin (Black and Hastings 1998). Therefore, it is suggested that composites are better candidates for tissue engineering, than homogenous materials. The properties of an implant can be tailored by controlling the volume fractions, and local and global arrangement of component phases (Ramakrishna, Mayer et al. 2001).

The underlying hypothesis of this project is that 'chitosan and PCL are versatile polymers with mutually complementary biomechanical properties and combining them would result in a unique biomaterial that can be fabricated into three-dimensional constructs with tunable physicochemical and biomechanical properties for a wide range of tissue engineering applications'.

1.4. SCOPE AND SPECIFIC AIMS: The ultimate goal of this project is to develop a novel composite biomaterial that can be fabricated into porous scaffolds with alterable mechanical, biological and degradation properties for the regeneration of a variety of soft tissues. This could be achieved by mixing chitosan and PCL for their unique contribution to the necessary biomechanical properties. However, chemical reactions such as copolymerization or crosslinking between chitosan and PCL may compromise their individual properties. Therefore, an ideal option is to make the polymeric components co-exist by mixing them physically. Physical mixing can be done in two ways: melt-mixing or dissolution in a common solvent. Melting the polymers is a poor option as it changes the physicochemical properties similar to chemical modifications, leaving solution mixing as the possible option. However, there is a lack of common solvent for chitosan and PCL as they are hydrophilic and hydrophobic by nature, respectively. Blending these two polymers therefore presents a unique challenge in this project. Additionally, chitosan and PCL mixtures present a model system for studying composites of two semi-crystalline polymers that differ in charge distribution, as chitosan is cationic in nature and PCL is uncharged.

The scope of this project is depicted in a schematic (**Figure 1.1)** where chitosan and PCL are to be combined and characterized in a sequence of steps, while studying the effects of this unique combination on properties vital to biomedical applications. The specific aims of this project are:

Specific aim 1: **To obtain a homogeneous mixture of chitosan and PCL that can be synthesized into 2D and 3D scaffold matrices.** This is a challenging task as chitosan is hydrophilic and insoluble in organic solvents whereas PCL is hydrophobic. Different methods of mixing and formation of membranes and scaffolds are explored (discussed in chapter 3).

Specific aim 2: To test and optimize the mechanical, bioregulatory, degradation and antibacterial properties of chitosan-PCL mixtures *in vitro*. Given the complementary nature of chitosan and PCL with respect to these properties, the conditions necessary for a composite with optimum qualities will be evaluated (discussed in chapters 4 through 7).

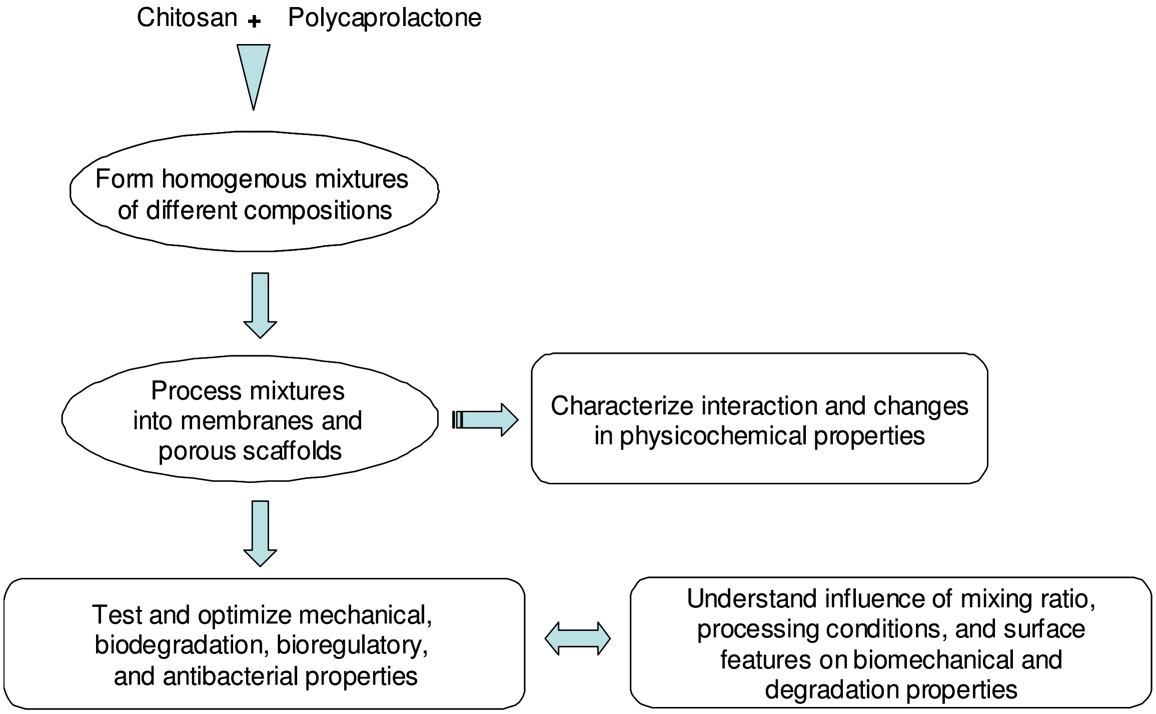

Figure 1.1. Schematic of Research Plan

Specific aim 3: To study the interaction between chitosan and PCL in the mixture at a molecular level. Miscibility of chitosan and PCL and changes in their physicochemical properties such as crystallinity and melting point will be evaluated in order to understand the mutual effects of mixing these uniquely different biomaterials (discussed in chapter 8).

Specific aim 4: **To understand the influences of mixing, processing conditions, and surface features.** This will be done simultaneously while testing the bio-mechanical and degradation properties of the composites (discussed in chapter 8).

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CHAPTER II

BACKGROUND

2.1. TISSUE ENGINEERING: In the past fifteen years, tissue engineering has emerged as an inter-disciplinary field, giving immense promise for the regeneration of virtually every type of tissue- liver (Mayer, Karamuk et al. 2000), bone (Rose and Oreffo 2002), muscle (Wright, Peng et al. 2001), cartilage (van der Kraan, Buma et al. 2002), cardiovascular tissue (Teebken and Haverich 2002), nerves (Evans, Brandt et al. 2002), intestines (Rocha and Whang 2004) and more. Tissue engineering inculcates knowledge of cell and molecular biology, biochemistry, pathology and clinical science, and the expertise of chemical engineering and materials science. The basic concept of tissue engineering is illustrated in **Figure 2.1** where cells of the target tissue type are isolated from the patient's own body or from donors, to be proliferated and modified *in vitro* (Langer and Vacanti 1993). These cells are seeded on a sterile porous scaffold fabricated from biocompatible and biodegradable materials in three-dimensional (3D) shape of the desired organ. Specific growth factors are added to the cell-seeded scaffold and incubated under optimum environmental conditions such as temperature and pH. Cells attach onto the scaffold, grow, spread and multiply, synthesizing the extracellular matrix (ECM). At this stage, the scaffold is implanted in the body at the site of the defective organ. Alternatively, the bare scaffold, loaded with growth factors is implanted in the body, where cells are recruited *in situ* from the surroundings and grown on the scaffold

(Langer and Vacanti 1993). The ECM organizes into a new tissue which integrates with the host tissue. During this time, the scaffold supports the new tissue, withstanding pressures from surrounding organs and fluids, and mechanical stresses. Slowly and steadily, as the new tissue is formed, the scaffold breaks into smaller fragments which eventually degrade into non-toxic products in the body.

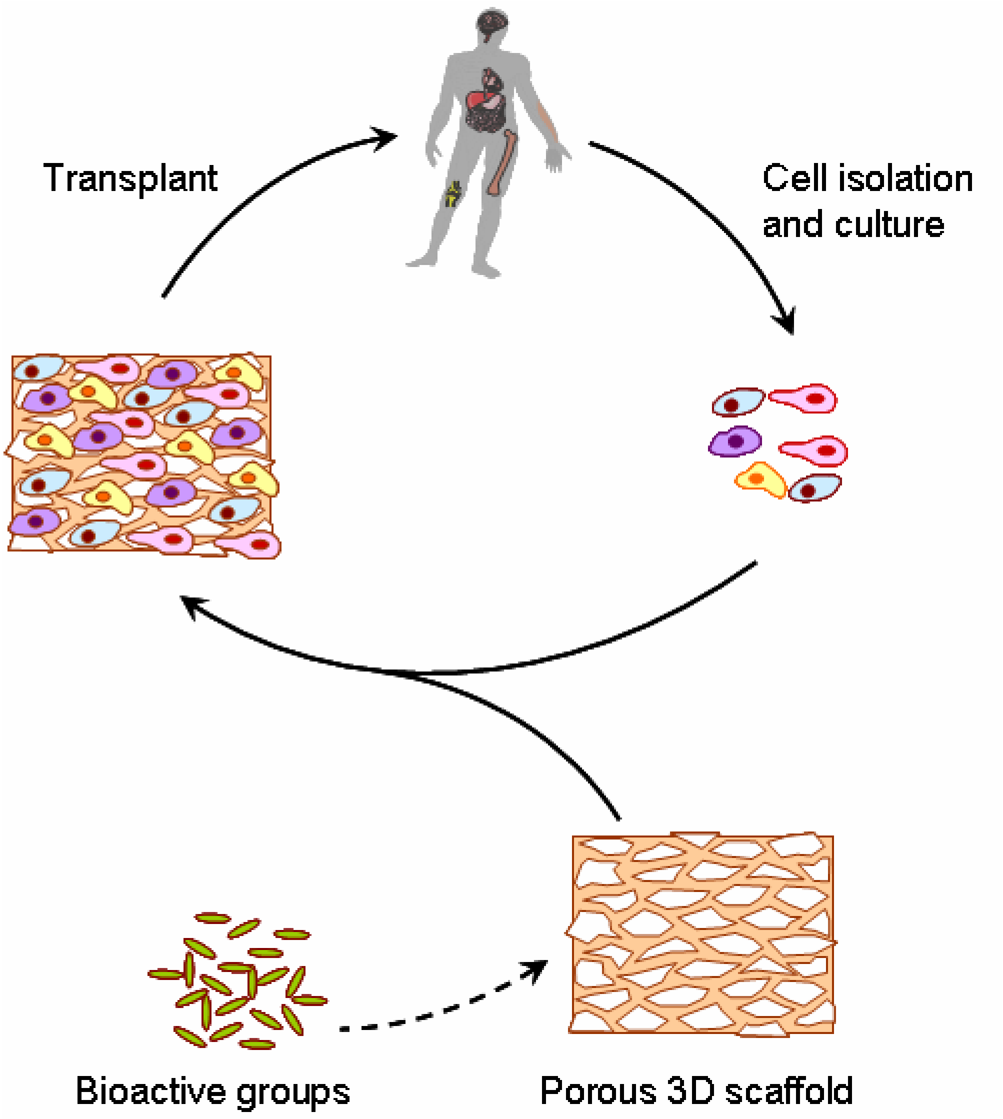

Figure 2.1. Concept of tissue engineering: cells of the desired tissue are seeded *in vitro* on a porous biocompatible scaffold, which is transplanted inside the body. Cells grow to form new tissue while the scaffold degrades away.

Degradation of scaffold material is facilitated by different mechanisms including hydrolytic reactions, enzyme-dependent reactions, and metabolic processes (Hutmacher 2000). The products of degradation are removed from the body by natural processes.

Using this approach, tissues of any type could be formed from the native cells. For example, osteoblast cells can be guided to form bone tissues (Meyer, Loos et al. 2004), hepatocytes for liver (Davis and Vacanti 1996), or chondrocytes for cartilage (Suh and Matthew 2000). The cell sources are usually autologous (patient's own body), but these are limited due to morbidity. In such situations, stem cells are excellent sources as they can be differentiated to form multiple cell phenotypes (Szilvassy 2003; Barry and Murphy 2004; Cortesini 2005). Stem cells are a healthy and renewable source of undifferentiated cells, found in the embryonic stage (Shufaro and Reubinoff 2004) as well as in adults (Short, Brouard et al. 2003; Szilvassy 2003). Currently, stem-cell based tissue engineering is being widely pursued (Barry and Murphy 2004; Wu, Liu et al. 2006).

Progress in tissue engineering has already made commercial skin available for treating patients with diabetic ulcers and burns (Integra). Tissue engineered bone and cartilage are expected to be available in near future. However, despite these advances, engineering three dimensional organs such as liver, heart, kidneys, and pancreas is still a distant dream. This is partly due to the complex structure and function of these organs, involving a multitude of physical, chemical and biochemical factors playing a simultaneous role and partly due to limitations of tissue engineering by itself.

The limitations of tissue engineering can be broadly classified into two groups (Levenberg and Langer 2004):

1. Defects in scaffolds, and the biomaterials used in their fabrication.

2. Insufficient knowledge of cell-material interactions.

2.2. SCAFFOLDS: Porous scaffolds are synthesized from biodegradable materials by different methods such as phase-separation (Madihally and Matthew 1999), gas foaming (Mooney, Baldwin et al. 1996), particulate-leaching (Mikos, Thorsen et al. 1994) and more recently, 3D printing (also known as solid freeform fabrication or rapid prototyping) (Zein, Hutmacher et al. 2002; Leong, Cheah et al. 2003). Important among these are phase-separation (also known as freeze-drying) and 3D printing. Freezing and lyophilization of polymer solutions at a controlled rate gives scaffolds with a defined pore structure and size (**Figure 2.2**) (Madihally and Matthew 1999).

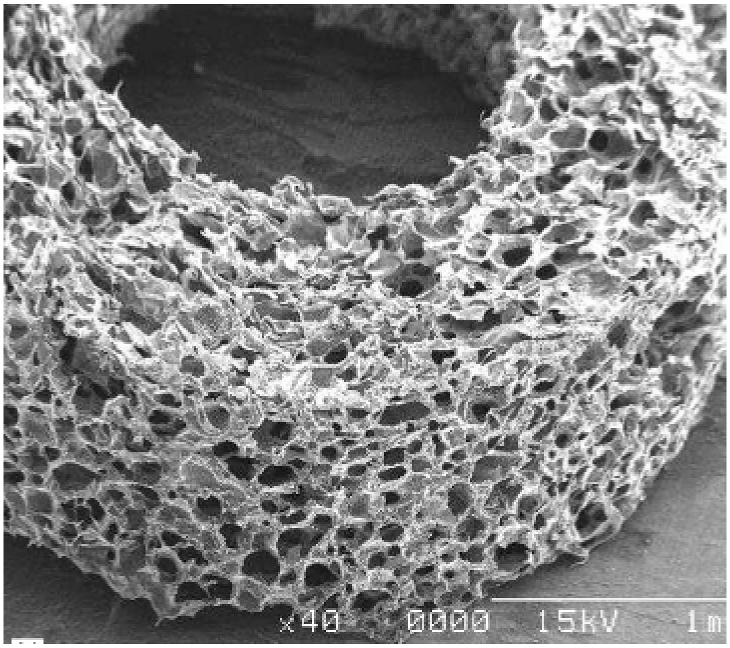

Figure 2.2. SEM image of a cylindrical porous scaffold of chitosan, obtained by controlled-rate freeze-drying process (Madihally and Matthew 1999).

In this process, polymers dissolved in suitable solvents are frozen to below -100°C. Lyophilization removes the solvents from a solid state to a direct gaseous state (sublimation) leaving behind a porous network in the scaffolds. The pore size may be controlled by altering the solvents or freezing temperatures. For example, dissolution of PLGA in benzene produced scaffolds with much smaller pores, compared to chloroform; and freezing at lower temperatures (-196°C) showed a similar effect (Moshfeghian, Tillman et al. 2006).

Freeform printing involves the extrusion of viscous polymer solutions through a fine needle in a computer-defined three-dimensional pattern (Leong, Cheah et al. 2003; Tsang and Bhatia 2004). Compared to freeze-drying, this process is much faster and has higher control over pore morphology but is more expensive. Particulate-leaching and gas-foaming are less commonly used techniques that involve the use of a salt or gas, respectively as a porogen (Mikos, Thorsen et al. 1994). In particulate leaching, water soluble salt particles such as NaCl are mixed with the polymer dissolved in an organic solvent. After casting and evaporation of the solvent, the salt is removed by leaching or dissolution in water. The amount and size of the salt particles determine the pore structure of the scaffold. The need for organic solvents is eliminated by forming discs of the polymer by heating and compression molding (Mooney, Baldwin et al. 1996). The discs are exposed to high pressure CO_2 which leaves a porous structure through the disc, but the pore structure has poor interconnectivity.

The basic criteria for designing tissue engineering scaffolds are (Hutmacher 2000; Ma 2004):

1) Structural and mechanical integrity to support the new tissue growth, while

withstanding stresses at the site of implant. Meet standard design criteria like strength, deformation, fatigue and wear.

2) Biodegradable at a rate matching that of neotissue formation. Faster degradation rates are undesirable as the new tissue will not be completely supported, leading to its collapse before integration with the host tissues.

3) High porosity and optimum pore size. Pore sizes are known to affect cell adhesion and spreading. Small pore sizes hinder the migration of cells, nutrients and wastes through the scaffold whereas large pores decrease the interconnectivity of the scaffold network. The optimum pore size differs among cell types, depending on the cell size. For example, higher porosity and smaller pore sizes increase the elasticity of the scaffold and provide higher surface area for cell attachment and ECM generation (Doi, Nakayama et al. 1996; Agrawal and Ray 2001).

These requirements are in addition to biocompatibility and bioactivity of the scaffold. That is, the scaffold should be non-toxic and non-immunogenic to the cells, and be able to regulate necessary cellular mechanisms such as adhesion, growth, migration, proliferation and differentiation (Hutmacher 2000). The specific criteria that govern the properties of a scaffold depend on the tissue of interest and the specific application. For example, in the vascular field, compliance matching of the implant and the host tissue is important (Surovtsova 2005), whereas in orthopedics, resistance to flexion, compression, and abrasion is important (Burg, Porter et al. 2000). Studies have shown that the physical forces also play a critical role in cell integrity and development (Goldmann 2002). Cells convert mechanical signals into biochemical responses through molecular mediators like integrins, focal adhesion proteins, and the cytoskeleton (Goldmann 2002). These molecules associate with the skeletal scaffold via the focal adhesion complex, when activated by cell binding to the extracellular matrix.

2.3. BIOMATERIALS: Construction of the scaffold, its architecture and pore morphology affect the mechanical strength, degradation rate and biological properties to some extent (Ma 2004). Majority of the strengths and drawbacks of scaffolds arise directly from the materials used for their fabrication. Therefore, key deficiencies in tissue engineering can be addressed by designing and developing biomaterials.

Biomaterials are biocompatible substrates with appropriate mechanical and chemical properties. They are of two types: inert and degradable. Inert biomaterials like poly(methylmethacrylate) are used in medical devices like sutures and catheters and they do not degrade (Wise 1995). Biodegradable polymers are the primary materials used for making scaffolds in various soft and hard tissue applications (Seal, Otero et al. 2001). Some applications require the substrate to degrade within minutes while others may range from hours to days (Wiseman, Domb et al. 1998). Polymeric biomaterials are of two types: natural and synthetic. Natural polymers are obtained from nature and have the biofunctional molecules necessary for cell regulation but they have limited mechanical properties. On the contrary, synthetic polymers offer excellent mechanical properties and processability of physicochemical properties, but they lack the necessary cell signaling molecules. The major biomaterials investigated in tissue engineering are discussed below.

2.3.1 Natural polymers

Collagen is the most abundant protein in animals, present in the connective tissues

(Alberts, Bray et al. 1994). Three polypeptide chains in the form of a triple helix constitute the structure of collagen. It has excellent biocompatibility and contains integrin-binding domains which control cell adhesion and shape (Lee, Singla et al. 2001). It is biodegradable by different matrix metalloprotenases (MMPs). It is used in cosmetics and drug delivery vehicles. However, the use of collagen is limited by its poor mechanical strength, which could be increased by crosslinking the fibers (Charulatha and Rajaram 2003). Hydrophilicity of collagen is also a drawback as it leads to swelling (Thacharodi and Rao 1995).

Gelatin is a thermally denatured form of collagen. It is primarily used as a gelling agent as it forms thermoelastic gels upon cooling to below 35°C (Ratner, Hoffman et al. 1996). It is also used in medical and pharmaceutical applications, owing to its biocompatibility and biodegradability (Young, Wong et al. 2005). Gelatin contains arginine-glycine-aspartic acid (RGD)-like amino acid sequence that promotes cell adhesion and migration (Mao, Zhao et al. 2003). It is also known to have a high hemostatic effect (Mao, Zhao et al. 2003). Similar to collagen, gelatin has poor mechanical properties when used alone, and is more suitable for hydrogels or drug microcarrier particles, rather than tissue engineering scaffolds (Young, Wong et al. 2005).

Sodium alginate is a derivative of alginic acid, a polysaccharide obtained from various species of brown seaweed (Drury and Mooney 2003). Like gelatin, alginate forms stable gels, which are used as drug delivery vehicles or as injectable cell-loaded hydrogels. Cell attachment, proliferation, and differentiation could be promoted by covalently coupling adhesion ligands to alginates (Alsberg, Anderson et al. 2001).

Starch is another polysaccharide found abundantly in plants. It is completely biodegradable and is explored in several biomedical applications (Marques, Reis et al. 2002). Composites of starch are more popular than starch itself (Mano, Sousa et al. 2004).

Glycosaminoglycans (GAGs) are the most abundant heteropolysaccharides in the body, present mainly on the surface of cells or as extracellular matrix (ECM) elements covalently bound to a core protein (Alberts, Bray et al. 1994). They are rigid, highly viscous and negatively charged molecules containing a repeating disaccharide unit. They provide structural integrity to cells and facilitate cell migration. The most important GAGs are heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, keratan sulfate and hyaluronic acid. However these biological polymers have poor mechanical properties and allow limited tailorability of physicochemical properties, in comparison to chitosan (see section 2.4).

2.3.2. Synthetic polymers

Synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), polyurethane and polycaprolactone (PCL) are used in various areas of medicine such as the cardiovascular system (Dacron and Teflon), orthopedics, and drug delivery systems (Griffith 2000). They can be easily reproduced with specific molecular weights, block structures, degradable linkages, and crossliking modes. These properties in turn, determine gel formation dynamics, crosslinking density, and material mechanical and degradation properties.

PGA, PLA, and their co-polymer PLGA have been investigated for more applications than any other degradable polymers. Extensive literature data exists on the technology to tailor the degradation and mechanical properties (Anderson and Shive 1997; Johansen, Men et al. 2000). Despite the strong approval history of PLGA polymers by FDA, a major disadvantage in using PLGA polymers lies in their lack of biological support to cellular adhesion, spreading, proliferation, and migration of most of the cell types (Massia and Hubbell 1990). A variety of chemical modifications have been attempted to improve the cell-substrate interactions of PLGA polymers including a) co-polymerizing with other synthetic polymers (Kwon, Park et al. 2002), and b) cross-linking with peptide sequences necessary for cellular attachment (Yang, Roach et al. 2001). Apart from cell-substrate interactions, a substrate has to mediate a variety of other signaling mechanisms such as growth factor activity, growth factor-cell interactions to regulate the biological response of diverse cell types (Giannobile 1996; Baldwin and Saltzman 1998). Recreating all the required biological responses is beyond the realistic possibilities in a synthetic system. In addition, degradation products of PLGA are relatively strong acids that may cause inflammation and massive swelling, threatening the structural stability of the scaffold (Agrawal and Athanasiou 1997). Aliphatic polyester PCL is an attractive candidate for tissue engineering, due to its easy processability and versatility in mixing with other polymers (Wiseman, Domb et al. 1998) (see section 2.5).

Recent developments in tissue engineering have focused on the design of biomimetic materials that are capable of eliciting specific cellular responses mediated by biomolecular recognition (Shin, Jo et al. 2003). This has been achieved by incorporating bioactive molecules such as ECM proteins and peptides by physical or chemical methods. The biomolecules interact with cell receptors and regulate many cellular activities including cell-matrix interaction and biodegradation. Nevertheless, several challenges facing this class of materials include design of adhesion molecules for specific cell types and lack of materials with mechanical properties matching those of living tissues.

2.4. CHITOSAN: Chitosan has been commercially used in waste water treatment due to its coagulating nature and high ability to adsorb and form polyelectrolytic complexes with metal ions (Babel and Kurniawan 2003; Varma, Deshpande et al. 2004; Crini 2005). Its excellent film forming capabilities coupled with anti-bacterial nature propelled its use as a wound dressing and food-packaging material (Shahidi, Arachchi et al. 1999; Khor and Lim 2003; Senel and McClure 2004). It is obtained from chitin, the second most abundant polysaccharide in nature, which is found in the exoskeletons of crustaceans such as crab and shrimp (Khor and Lim 2003). Chitin comprises of a linear chain of N-acetyl D-glucosamine molecules. Deacetylaton of chitin to various degrees (typically 60-95%) in hot concentrated alkali removes the acetyl groups and forms chitosan. Therefore, chitosan maybe considered as a copolymer of D-glucosamine and N-acetyl D-glycosamine residues where the relative ratio of D-glucosamine to N-acetyl D-glucosamine represents the degree of deacetylation of chitosan (**Figure 2.3**). The chemical structure of chitosan has been investigated by Fourier Transform Infrared Spectroscopy (FTIR) in which functional groups of a molecule absorb infrared radiation at specific wave numbers. A typical FTIR interferogram of chitosan shows the amine and hydroxyl functional groups which show overlapping peaks in 3300-3500cm^{-1} range (Pawlak and Mucha 2003) (similar to results shown in Figure 8.1A)

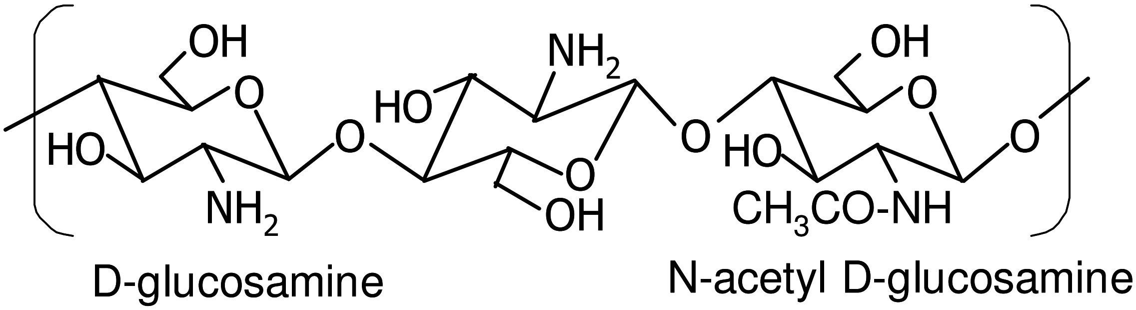

Figure 2.3. Molecular structure of chitosan. Chitosan is obtained by partial deacetylation of a polysaccharide, chitin.

Chitosan dissolves in dilute acids (pH<6.3), with a dissociation constant (pKa) of 6.2-7.0 (Bodnar, Hartmann et al. 2005). In solution form, the amine group protonates and provides a net positive charge on chitosan. The cationic nature of this polyelectrolyte is critical to its coagulating nature and interaction with negatively charged serum proteins, collagen and glycosaminoglycans present in the extracellular matrix of cells. It swells in neutral and alkaline media and forms gels, a property that can be used to make pH sensitive hydrogels and controlled-release systems (Kumar 2000; Berger, Reist et al. 2004; Berger, Reist et al. 2004).

Chitosan is semi-crystalline by nature, which means that it has both crystalline and amorphous regions (Suyatma, Tighzert et al. 2005). However, the temperature at which the transition from amorphous to crystalline phase takes place (glass transition temperature, T_g) is unclear due to contradictory reports in literature. Also, transition temperatures may vary significantly based on thermal history of the sample. The phase transitions of chitosan membranes investigated by Differential Scanning Calorimetry (DSC) shows a typical thermogram obtained from first scan (Kittur, Prashanth et al. 2002) (similar to results shown in Figure 8.3). The exothermic peak at 280°C corresponds to thermal decomposition of chitosan which takes place before melting transition. The endothermic peak at 130°C indicates the evaporation of residual or water.

The crystal structure of chitosan has been investigated by Wide Angle X-ray Diffraction (WAXD). A typical WAXD pattern of chitosan exhibits crystal structure with clear peaks at diffraction angles (2θ) of 10° and 20.5° (Lee, Kim et al. 2000) (similar to results shown in Figure 8.2A**).** The MW and DD of chitosan have a major influence on its physicochemical properties (Berth, Dautzenberg et al. 1998). The Mark-Houwink equation $[\eta]= k.\ M^{\alpha}$ is used to correlate viscosity (η) and MW (M) of polymers, where k and α are constant values for a given material (Wang, Bo et al. 1991). As DD increased from 69% to 100%, the values of *k* increased from 0.104*10-3 to 16.8*10-3ml/g and those of α decreased from 1.12 to 0.81. Therefore, it can be said that the MW of chitosan also varies with DD.

Derived from a natural source, chitosan has limited mechanical properties. In a dry state, chitosan films have a tensile strength of 30MPa with 3-5% strain (Singh and Ray 1997). Similar to other hydrophilic materials, wetting increased the elongation of chitosan from 5 to 70% but lowered the modulus from 1250 to 6MPa (Cheng, Deng et al. 2003). Increasing the MW from 240 to 410kD increased the tensile strength of chitosan from 8 to 23MPa and elongation from 50 to 110% (Chen and Hwa 1996).

Chitosan is biodegradable and is enzymatically broken down into nontoxic monosacchrides such as D-glucosamines by the action of lysozymes present in the body as well as chitosanase (Shigemasa, Saito et al. 1994; Fukamizo, Honda et al. 1995). The degradation rate is dependent on several factors including MW, DD, local pH, temperature, structural stability (mechanical properties) and homogeneity of source

(Davies, Neuberge.A et al. 1969; Pangburn, Trescony et al. 1982; Shigemasa, Saito et al. 1994). Previous research has shown that highly deacetylated chitosan is less susceptible to lysozyme and that chitosan prepared homogenously degrades faster than that prepared heterogeneously (Pangburn, Trescony et al. 1982; Shigemasa, Saito et al. 1994). At very high degree of deacetylation, adsorption of lysozyme does not result in the depolymerization of chitosan (Kristiansen, Varum et al. 1998). Thus, while using chitosan in tissue engineering applications, increased deacetylatation decreases biodegradation by lysozyme (Nordtveit, Varum et al. 1996; Tomihata and Ikada 1997; Mi, Tan et al. 2002). In addition, lysozymal hydrolysis of chitosan is at its highest in acidic conditions (pH 4.5-5.5) (Nordtveit, Varum et al. 1996). Nevertheless, degradation can also be influenced by mixing with other polymers.

Chitosan is known for its excellent biocompatibility, which could be attributed to its source chitin, which is obtained from nature. Chitosan has a net positive charge that binds with anionic cell surface, and thus regulates their adhesive and proliferative activity(Chupa, Foster et al. 2000). Although chitosan has no specific binding domain for integrin-mediated adhesion, it supports biological activity of diverse cell types and it has been a subject of many investigations in tissue engineering (Chupa, Foster et al. 2000; Lahiji, Sohrabi et al. 2000; Cai, Yao et al. 2002; Chung, Yang et al. 2002; Zhu, Ji et al. 2002; Mizuno, Yamamura et al. 2003). Especially, chitosan based scaffolds have favored the proliferation of chondrocytes, showing tremendous promise for regenerating cartilage tissues (Suh and Matthew 2000). Hemocompatibility is another advantage of chitosan which allows its use in coating stents and other medical devices (Rao and Sharma 1997). Layering bioprosthetic valves with chitosan is an option as it inhibits calcification

(Kuribayashi, Chanda et al. 1996). Chitosan can also accelerate wound healing by regulating the inflammatory pathway (Hwang, Chen et al. 2000; Vachoud, Chen et al. 2001), and macrophage metabolism (Porporatto, Bianco et al. 2003) which are crucial in the body's defense program.

Chitosan is known for its bacteriostatic and bactericidal nature against numerous pathogenic organisms including *Porphyromonas. gingivalis* (Ikinci, Senel et al. 2002), *Escherichia coli* (Chung, Wang et al. 2003), *Actinobacillus actinomycetemcomitans (Choi, Kim et al. 2001), Pseudomonas aeroginosa, Staphylococcus aureus* (Chung, Wang et al. 2003) and *Streptococcus mutans (Tarsi, Muzzarelli et al. 1997; Choi, Kim et al. 2001).* Therefore, it has been explored as wound dressing and coating medical devices to help prevent infection (Khor and Lim 2003). The same cationic nature that helps chitosan bind with cells and regulate their activity is believed to break bacterial cell walls, this causing death (Choi, Kim et al. 2001; Helander, Nurmiaho-Lassila et al. 2001; Qi, Xu et al. 2004). Further, increased MW and DD of chitosan increase the potency of antibacterial activities (Chen, Chung et al. 2002). Chitosan exhibits stronger antibacterial property towards gram-positive bacteria than gram-negative bacteria (No, Lee et al. 2003).

In summary, chitosan has the best mechanical properties among all the existing natural polymers, with tunable physicochemical and degradation properties. Its biocompatibility and bioregulatory properties, coupled with antibacterial nature make it an excellent candidate for tissue engineering.

2.4.1. Limitations of chitosan: Despite the advantages stated in the previous section, primarily chitosan lacks the mechanical properties required for regeneration of many tissues. For example, the tensile strength of an articular cartilage is around 27MPa (Black and Hastings 1998), whereas chitosan has shown a strength of only 5-7MPa, in the wet state (Cheng, Deng et al. 2003). The rationale for combining chitosan with other polymers has been diverse and application-specific. In making hydrogels for drug delivery, the primary concern was to influence the network structure and pH dependent swelling behavior (synonymous with hydrophilicity) and degradation kinetics of chitosan gels (Lee, Kim et al. 2000). In wound dressings and food packaging products, enhancing the mechanical properties and antimicrobial nature of chitosan films maybe the main objective. In tissue engineering, tunability of biomechanical and degradation properties, of chitosan is important, because of the wide range of properties exhibited by natural tissues. For example, soft tissues such as skin has a strength of 7MPa, whereas a cortical bone has a strength of 133MPa, measured in the longitudinal direction (Black and Hastings 1998). Similarly, the spreading and proliferation kinetics of fibroblasts are different from that of chondrocytes.

2.4.2. Review of chitosan mixtures: Blending two polymers is an approach to develop new biomaterials exhibiting combinations of properties that could not be obtained by individual polymers. For example, the poor cell adhesion normally associated with poly (2-hydroxyethylmethacrylate) was mitigated by blending with gelatin (Santin, Huang et al. 1996). Chitosan was combined with other polymers either by physical methods such as solution mixing (Olabarrieta, Forsstrom et al. 2001) or melt mixing (Correlo, Boesel et al. 2005), or by chemical modifications such as grafting (Liu, Li et al. 2004), copolymerization (Liu, Li et al. 2005), complexation or cross-linking (Shanmugasundaram, Ravichandran et al. 2001; Kulkarni, Hukkeri et al. 2005).

Synthetic polymers impart their excellent mechanical properties, processability and tunability of physicochemical properties to polymer systems. For example, the hardness of chitosan membranes was reduced by mixing with crystalline polymers polyvinyl alcohol, polyethylene oxide and polyethylene glycol (Arvanitoyannis, Kolokuris et al. 1997; Khoo, Frantzich et al. 2003; Kolhe and Kannan 2003). Swelling of chitosan can be reduced by combining with Poly(lactic acid) (PLA) and Poly(lactic-co-glycolic acid) (PLGA) (Nam and Lee 1997; Perugini, Genta et al. 2003). On the other hand, biological molecules such as collagen and GAGs are used to influence specific cellular activities such as adhesion and proliferation, especially in tissue engineering (Chupa, Foster et al. 2000). Heparin, a GAG is known to minimize platelet adhesion on chitosan (Wang, Li et al. 2003). In other cases, chitosan was added to increase the affinity of cells to synthetic surfaces that lack hydrophilicity and/or cell recognition sites (Wu, Shaw et al. 2006). The processability and use of chitosan is hampered mainly by its insolubility in most organic solvents, restricted solubility in water ($pH<6.2$) and poor reactivity.

The physicochemical properties of chitosan have been modified by grafting various types of side chains onto the amine or hydroxyl functional groups of chitosan backbone (Jayakumar, Prabaharan et al. 2005). These functionalized derivatives of chitosan were found to have improved solubility, anti-microbial activity, anti-oxidant activity, complexation, adsorption and chelation properties while retaining some of the beneficial properties of chitosan, such as mucoadhesivity, biocompatibility and biodegradation. Most graft copolymers improved the solubility of chitosan at different pH conditions and hence are useful for drug delivery applications. One of the problems associated with graft copolymerizaton of chitosan is the loss of amine functional group which imparts chitosan its unique cationic and biological nature. Copolymerization taking place at random amine group sites on the chitosan backbone blocks their availability. Therefore regioselective grafting of polymer chains onto chitosan is suggested as a better alternative than graft copolymerization (Jayakumar, Prabaharan et al. 2005).

2.5. POLYCAPROLACTONE: PCL is one of the most flexible and easy to process polymers, but also one of the slowest degrading ones (Wiseman, Domb et al. 1998). It is non-toxic and tissue compatible, and is mostly used in controlled drug release and sutures. Other applications are suture coatings, absorbable medical devices like intravascular stents as support to vein grafts, and as external grafting material for broken bones.

PCL is a semi-crystalline, aliphatic polyester obtained by ring-opening polymerization of ε-caprolactone (Yu and Liu 2004). ε-caprolactone consists of 5 nonpolar methylene groups and single relatively polar ester group which gives it unique properties (**Figure 2.4**). These functional groups are evident from FTIR analysis (Elzein, Nasser-Eddine et al. 2004) (similar to results shown in Figure 8.1A). PCL has a low melting (59 to 64°C) and glass transition temperatures (-60°C) (Wang, Cheung et al. 2002). It has a thermal decomposition temperature T_d of 350°C, which is much higher than that of other aliphatic polyesters (235-255°C) (Wise 1995). Unlike most aliphatic polyesters, PCL is always in rubbery state at room temperature (due to its low T_g) which makes it highly permeable for many therapeutic drugs. The crystal structure of PCL as investigated by WAXD shows sharp peaks at 21.5°, 22° and 23.5° (Gan, Liang et al. 1997) (similar to results shown in Figure 8.2B).

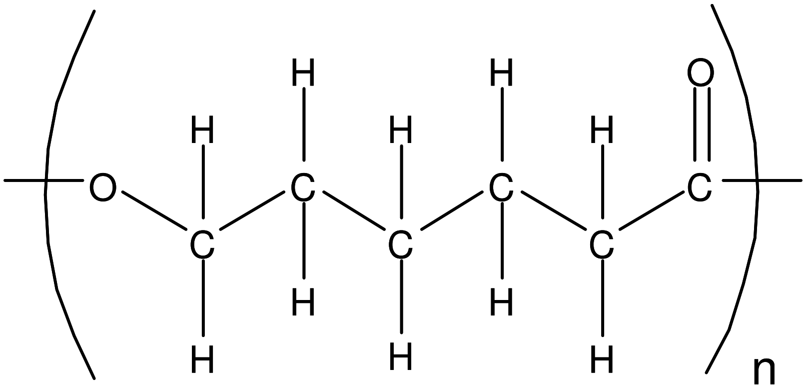

Figure 2.4. Molecular structure of Polycaprolactone

The mechanical properties of PCL are similar to polyolefins due to high olefinic content (Wiseman, Domb et al. 1998). The values reported in literature for PCL with MW 44kD are: tensile strength 16MPa, tensile modulus 400MPa, flexural modulus 500MPa, elongation at yield 7.0% and elongation at break 80% (Ratner, Hoffman et al. 1996). Compared to PLGA, PCL has lower stress and modulus values but significantly higher extensions.

Hydrolytically unstable aliphatic- ester linkage makes it biodegradable (Wiseman, Domb et al. 1998). PCL degrades slower than poly(α- hydroxy acids) like polylactide due to combination of its high crystallinity and olefinic character. PCL is susceptible to degradation by certain bacteria, yeast and fungi (Wiseman, Domb et al. 1998). Bioerosion of PCL (solubilization in water) is of two types: bulk (rate of water penetration exceeds rate at which polymer transforms into water-soluble material) and surface (Ratner, Hoffman et al. 1996). Especially, during enzymatic surface erosion, low MW fragments of PCL are taken up by macrophages and degraded intracellularly. Water absorption is lower compared to PLGA (Wise 1995).

Mechanical and degradation properties of PCL can be altered by regulating its crystallinity and molecular weight (Jenkins 1977). Mixing and copolymerization reduce crystallinity thereby increasing accessibility of ester linkage and enhancing rate of hydrolysis i.e. degradation (Wiseman, Domb et al. 1998). Hence PCL blends/copolymers can be made to have varying mechanical and physical properties and rates of degradation.

PCL is also a biocompatible polymer i.e. non-toxic and non-inflammatory (Wiseman, Domb et al. 1998). Hence, it has been approved by the FDA and is used in sutures. However, PCL lacks the ability to regulate specific cellular processes such as adhesion, spreading and differentiation. This is probably due to it's a) hydrophobicity, b) uncharged nature and c) lack of cell-binding domains.

2.5.1. Limitations of polycaprolactone: Primarily, PCL has a very low degradation rate which limits its use to controlled rate dug delivery (Wiseman, Domb et al. 1998). It has been shown that PCL degrades very slowly until the MW is reduced to 15kD (Cha and Pitt 1990). It also lacks cell-binding domains due to which its bioregulatory properties are also limited. But PCL can be easily combined with other polymers. Biomacromolecules such as starch can be immobilized on PCL to improve its cytocompatibility and biodegradation (Koenig and Huang 1995). Degradation rate can also be enhanced by lowering the molecular weight of PCL.

2.6. CELL-MATERIAL INTERACTIONS: Incomplete and insufficient understanding of cell-material interactions maybe cited as the second major drawback of tissue engineering (Hutmacher 2000). Majority of the cell-material interactions have been studied on two dimensional (2D) matrices which cannot be directly translated to (three dimensional) 3D matrices (Huang, Siewe et al. 2006). 3D matrices provide more space for cell attachment, extracellular matrix organization and cell-cell interaction. On the other hand, cells cultured on 2D matrices spread on a single flat plane. Surface topography, compliance, mechanical and degradation properties of 2D and 3D matrices are also very different. The simultaneous influence of these factors on cellular colonization in 3D matrices is not completely understood.

2.7. CHITOSAN AND PCL MIXTURES: Correlo et al. have mixed chitosan and PCL by melting and injection molding, but this reduced elongation of PCL from 670 to 5% (Correlo, Boesel et al. 2005). There was no change in crystal structure of PCL. However, the use of halogenated solvents is undesirable for toxic reasons. Wu has grafted chitosan and PCL, which resulted in reduced water absorptivity and weight loss by degradation (Wu 2005). Mixing aqueous solutions of chitosan with PCL solutions in chloroform resulted in reduced water vapor and oxygen permeability (Olabarrieta, Forsstrom et al. 2001). Grafting copolymerization of chitosan and PCL also suppressed the crystallinity of both the polymers (Liu, Li et al. 2004). This project aims at mixing chitosan and PCL in a non-halogenated, non-toxic solvent, thus preserving their unique individual properties.

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CHAPTER III

PREPARATION OF CHITOSAN AND PCL MIXTURES

3.1. INTRODUCTION

In tissue engineering, the lack of suitable biomaterials could be eliminated by combining natural and synthetic polymers, for their individual contribution to biomechanical properties of the implant. Composites of polymers can be prepared in several ways including solution mixing (Olabarrieta, Forsstrom et al. 2001), melt mixing (Correlo, Boesel et al. 2005), side chain grafting (Liu, Li et al. 2004), graft copolymerization (Liu, Li et al. 2005) and cross-linking (Shanmugasundaram, Ravichandran et al. 2001; Kulkarni, Hukkeri et al. 2005). The choice of method depends on the properties of the individual polymers as well as the desired product.

When a functional group is desired to be added to a non-functional polymer, grafting or graft-copolymerization of a functional polymer is favored. However, grafting on chitosan occurs on the amine groups, which is undesirable as the amine groups are responsible for the unique cationic and biological properties of chitosan (Muzzarelli and Peter 1997). Chemical crosslinking of polymers is undesirable due to toxicity problems associated with the crosslinking agents. Melt-processing of polymers is also a poor option as the physicomechanical properties of the components might be altered after high heat treatment. Therefore, solution mixing is an attractive choice for combining chitosan and PCL in this project, as it allows the polymeric components to co-exist without any chemical modification, thus retaining their individual properties. However, there is a lack of common solvent for chitosan and PCL as they are hydrophilic and hydrophobic by nature, respectively. Recent studies have reported the use of hexafluoroisopropanol (HFIP) for common dissolution of chitosan and PCL (Senda, He et al. 2002). However, halogenated hydrocarbons are toxic or carcinogenic to the body and their use should be minimized (Nielsen, Abraham et al. 1996). Therefore, obtaining a non-halogenated common solvent for chitosan and PCL is a unique challenge in this project.

In this work, different solvents and methods of mixing chitosan and PCL in a single phase are explored. The feasibility of various techniques and processing conditions for preparing membranes and porous scaffolds of these mixtures are tested. Some of these techniques are air-drying and oven-drying for membranes, and freeze-drying (Madihally and Matthew 1999) and freeze-extraction (Ho, Kuo et al. 2004) for scaffolds. The effects of relative compositions of chitosan and PCL are also studied by mixing them in three different mass ratios.

3.2. MATERIALS AND METHODS

Chitosan (85% DD and >310kD) and poly(ε-caprolactone) (PCL) (MWs 40kD and 80kD) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals used were of reagent grade. Chitosan and PCL were mixed in 75:25, 50:50 and 25:75 mass ratios, and were denoted as 25% PCL, 50% PCL and 75% PCL, respectively. These mixtures were prepared in three ways by dissolving the polymers either in aqueous acetic acid (25% or 77%) or in a ternary solvent system, as shown in **Table 3.1**. The table shows the different variables that were tested, such as solvent composition, drying temperature for forming membranes and scaffold formation method employed.

Table 3.1. Parameters tested in mixing chitosan and PCL, and forming membranes and porous scaffolds.

S.No.	Common dissolution solvent	Membrane drying conditions	Scaffold formation
1.	25% acetic acid	Room temperature	Freeze-drying
2.	77% acetic acid	1. Room temperature 2. 55°C in water bath 3. 55°C in oven 4. 55°C in oven followed by chloroform annealing	1. Freeze-drying 2. Freeze-gelation/ Freeze-extraction
3.	Ternary solvent system	Room temperature	-

3.2.1. Dissolution in 25% acetic acid: Three milliliters of chitosan dissolved in 0.5M acetic acid was taken in a glass vial and 1mL of PCL (MW 40kD) dissolved in glacial acetic acid was added to it, and stirred vigorously. The concentrations used were 1.8% of PCL with 1.8%, 0.6% and 0.2% of chitosan for 25% PCL, 50% PCL and 75% PCL mixtures, respectively. A precipitate of PCL was formed in all mixtures, in increasing amounts at higher PCL compositions. Half a milliliter (0.5mL) of chloroform was added to dissolve the PCL precipitate and vortexed to form a single phase solution. Membranes were formed at room temperature (**Section 3.2.4.a**) and scaffolds were formed by freeze-drying (**Section 3.2.5.a**).

3.2.2. Dissolution in 77% acetic acid: Three milliliters of chitosan dissolved in 0.5M acetic acid was taken in a glass vial and 10mL of PCL (MW 80kD) dissolved in glacial acetic acid, was added to it. The concentrations used were 1% chitosan with 0.1%, 0.3% and 0.9% of PCL for 25% PCL, 50% PCL and 75% PCL mixtures, respectively. The mixtures were stirred for two minutes and slightly warmed to obtain homogenous solutions. There was no phase separation even after one day at room temperature. Membranes were formed at different conditions (**Section 3.2.4**) and scaffolds were formed by different methods (**Section 3.2.5**).

3.2.3. Dissolution in ternary solvent system: Chitosan and PCL (MW 80kD) were dissolved in a ternary solvent system, composed of chloroform, acetic acid and water. This was achieved by first obtaining a single phase solution of the three solvents. It is known that acetic acid is completely miscible in chloroform and water. But chloroform and water are mutually immiscible. Therefore, the binodal plot of these three solvents at 25°C was plotted in SigmaPlot (SPSS Science, Chicago, IL) using data retrieved from CHEMCAD software (Chemstations, Inc., Houston, TX) (**Figure 3.1**). The corners of this triangle represent the pure phases, and the area enclosed under the plot indicates equilibrium compositions at 25°C. Literature values report miscibility of chloroform, acetic acid and water at 25°C at three compositions (**Table 3.2)** (Christensen, Gmehling et al. 1984). These compositions are indicated in blue color on the binodal triangular plot (**Figure 3.1**). Based on the molecular weights and density at 25°C, the volumes corresponding to equilibrium mole fractions of chloroform, acetic acid and water were calculated (**Table 3.2)** and mixed to obtain ternary mixtures.

Chitosan (MW >310kD) and PCL (MW 80kD) were added in equal mass ratios to each of the three ternary mixtures and stirred at room temperature for 2 minutes to obtain homogenous solutions. There was no phase separation even after one day at room temperature. Membranes were formed at room temperature (**Section 3.2.4.a**).

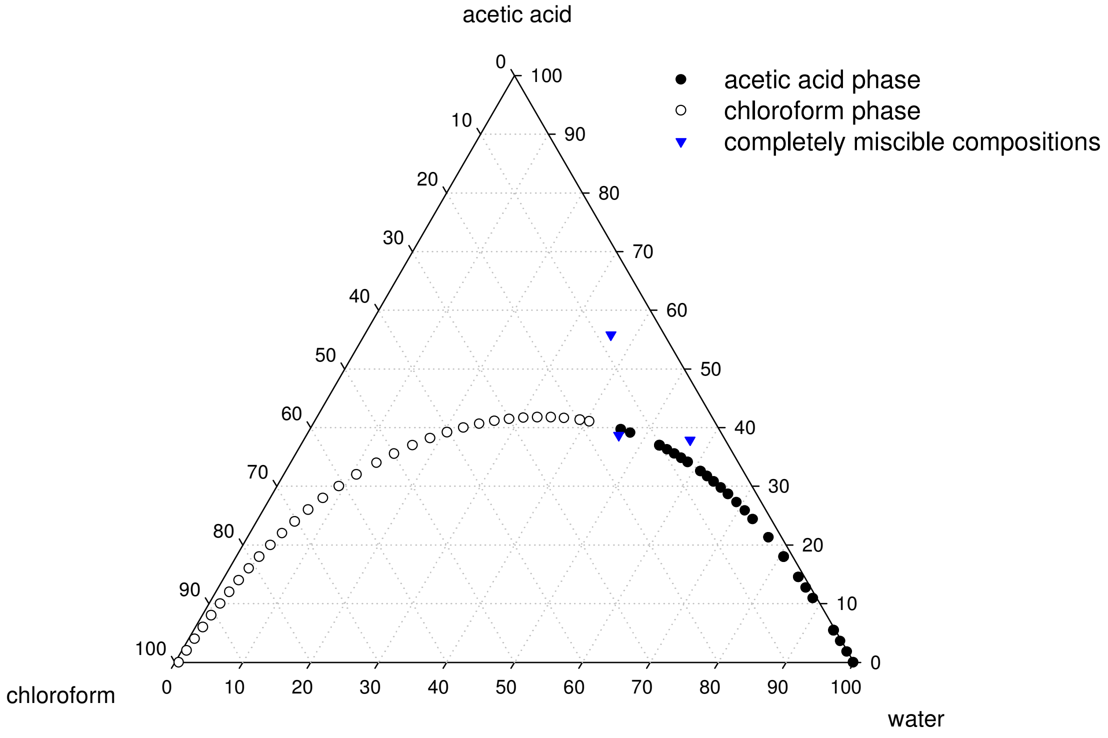

Figure 3.1. Binodal plot of chloroform, acetic acid and water at 25°C. Compositions are represented in mole %. Data points in blue color indicate equilibrium compositions (Christensen, Gmehling et al. 1984).

Table 3.2. Miscibility of ternary mixtures of chloroform, acetic acid and water at 25°C in mole fractions (Christensen, Gmehling et al. 1984). Equilibrium volumes were calculated from corresponding molecular weights and density at 25°C.

Mole fractions			**Volume (milliliters)**		
Chloroform	**Acetic acid**	**Water**	**Chloroform**	**Acetic acid**	**Water**
0.079	0.558	0.363	6.4	31.9	6.5
0.153	0.387	0.460	12.3	22.1	8.3
0.052	0.379	0.569	4.2	21.7	10.2

3.2.4. Formation of membranes: Fifteen milliliters of solutions were poured into Teflon dishes of 50mm diameter and dried overnight by the following three methods to form membranes of 80-100μm thickness:

a) At room temperature under the fume hood

b) At 55°C in a water bath (saturated humidity conditions)

c) At 55°C in an oven (unsaturated humidity state)

d) Membranes obtained from the oven at 55°C were solvent annealed by exposing them to an enclosed environment saturated with chloroform vapors at room temperature for 20 seconds.

3.2.5. Formation of porous scaffolds: Three dimensional porous scaffolds were made by three methods:

a) <u>Freeze-drying:</u> Three to five milliliters of solutions were taken in Teflon vials and frozen at -196°C by dipping in liquid nitrogen for 1 minute. Frozen vials were lyophilized overnight at -80°C in Virtis Lyophilizer (Virtis, Gardiner, NY) to obtain porous scaffolds.

b) <u>Freeze-extraction:</u> PCL and chitosan-PCL mixture scaffolds were formed by freeze-extraction method. Chitosan-PCL mixture solutions were taken in a 24-well tissue culture plate, 0.5mL each, and PCL dissolved in chloroform (not acetic acid) was taken in glass vials. All solutions were frozen at -80°C for 48 hours. After 48 hours, acetone precooled to -20°C was added to immerse the scaffolds and extract the solvent. Acetone was replaced every 6 hours and removed completely after 5 replacements. Formed scaffolds were dried at room temperature for 24 hours under a fume hood.

c) Freeze-gelation: This method is used to form scaffolds of chitosan. Solutions of chitosan were taken in a 24-well tissue culture plate, 0.5mL each and frozen at -80°C for 48 hours. After 48 hours, the tissue culture plate was transferred to a freezer (-20°C) and 1mL of NaOH (5N/ 200gm/L) solution precooled to -20°C was added to each well to gel the chitosan. NaOH was replaced every 6 hours and removed completely after 5 replacements. Formed chitosan gels were washed by adding distilled water to remove excess NaOH, and dried at room temperature for 24 hours under a fume hood.

3.2.6. Evaluation of membrane morphology: All membranes were qualitatively assessed for uniformity, continuum, phase separation, thickness, flexibility, transparency, smoothness and dimensional stability by visual inspection. Thickness of the membranes was measured by imaging the membrane cross-section followed by image analysis using Sigma Scan Pro software (SPSS Science, Chicago, IL), the details of which are explained elsewhere (Raghavan, Kropp et al. 2005).

3.2.7. Evaluation of scaffold pore morphology: All porous scaffolds were first assessed qualitatively for structural and dimensional stability by visual inspection and handling. For quantitative evaluation of pore morphology, samples were sectioned at various planes, attached to aluminum stubs with carbon paint and sputter-coated with gold for 1 minute. Prepared samples were then observed and imaged by JOEL 6360 Scanning Electron Microscope (Jeol USA Inc., Peabody, MA) at an accelerating voltage of 15kV. Using image analysis software Sigma Scan Pro (SPSS Science, Chicago, IL), these micrographs were analyzed for pore size, shape, and orientation as described in detail elsewhere (Moshfeghian 2005).

3.3. RESULTS

3.3.1. Influence of solvents and mixing on membrane morphology: Chitosan is insoluble in most organic solvents but dissolves in water at pH<6.4. Chitosan membranes formed from acidic water were smooth, transparent and soft. Increasing the molecular weight of chitosan gave tougher and softer membranes. On the other hand, PCL is hydrophobic and dissolves in a number of organic solvents. PCL is readily soluble in chloroform but dissolves slowly in acetic acid. PCL dissolved in glacial acetic acid gave white, opaque, smooth and tough membranes. On the other hand, PCL membranes cast after dissolving in chloroform were smooth, opaque, strong, and flexible.

When chitosan and PCL were dissolved in ternary equilibrium solvents of chloroform, acetic acid, and water, membranes formed at room temperature were uneven and phase-separated.

3.3.2. Influence of processing conditions and mixing on membrane morphology: Mixture membranes were first made by drying solutions at room temperature 25°C or at 37°C in a warm room with controlled humidity environment. Formed membranes were non-uniform and randomly separated into a smooth transparent phase (corresponding to chitosan) and a rough non-transparent phase (corresponding to PCL), despite the solutions being homogenous. Only 50% PCL membrane showed uniform dispersion of two polymers throughout the membrane.

Next, solutions were dried at 55°C (closer to the melting point of pure PCL) in an open water bath or in an oven. Membranes formed in the water bath were structurally more uniform with lesser phase separation compared to membranes dried at 25°C or at

37°C. However, the two phases appeared to have dried as two separate membranes stuck to each other. On the other hand, membranes formed in the oven were highly uniform in structure with negligible phase separation and had a unique morphology than the membranes formed by previous methods (**Figure 3.2**). Increased PCL content decreased the transparent nature of chitosan membranes.

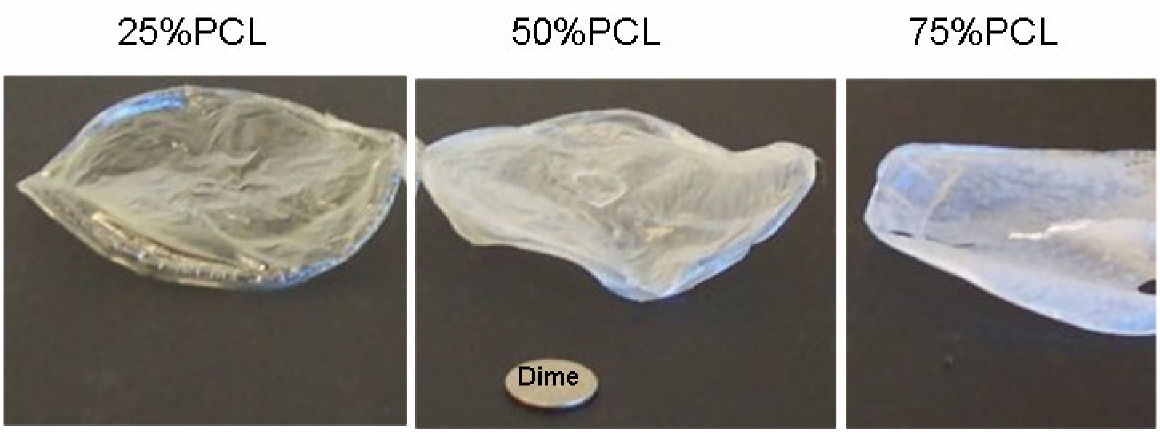

Figure 3.2. Influence of mixing chitosan and PCL in 77% acetic acid, on morphology of membranes formed in the oven at 55°C.

When the oven-dried membranes were annealed by exposing to chloroform vapors in an enclosed chamber, there were no changes in the phase distribution or uniformity of the membranes. However, membranes were softer and sticky immediately upon removal from the chamber, due to chloroform which acted as a plasticizer. As time elapsed beyond 5 minutes after removal from the chamber, the membranes lost their stickiness, probably due to the evaporation of chloroform.

3.3.3. Influence of solvents on scaffold pore morphology: Using freeze-drying method, mixture solutions in 25% or 77% acetic acid solvents did not form cylindrical structures mimicking the shape of the Teflon vials as they lacked structural integrity (**Figure 3.3**). However, their cross-section showed open pore morphology **(Figure 3.4)**. Chitosan formed a highly interconnected porous network with pore sizes ranging from 10-100µm. At low compositions of PCL, the inter-connectivity of pure chitosan scaffolds is retained, although there is an appearance of PCL globules in the network. As the PCL content is increased, the fallout or precipitation of the PCL globules is more pronounced. The scaffold with 75% PCL composition lost most of the inter-connectivity due to presence of large PCL globules. 50% PCL scaffolds showed a good balance of interconnectivity of chitosan and evenly dispersed PCL globules of uniform size.

Freeze-extraction process gave hard white disks of PCL, the hardness varying with concentration and MW of PCL. Scaffolds of chitosan-PCL mixtures dissolved or disintegrated during last stages of solvent extraction with acetone. Chitosan scaffolds formed by freeze-gelation were smooth jelly-like matrices, but they turned white and powdery after washing off the excess NaOH and drying.

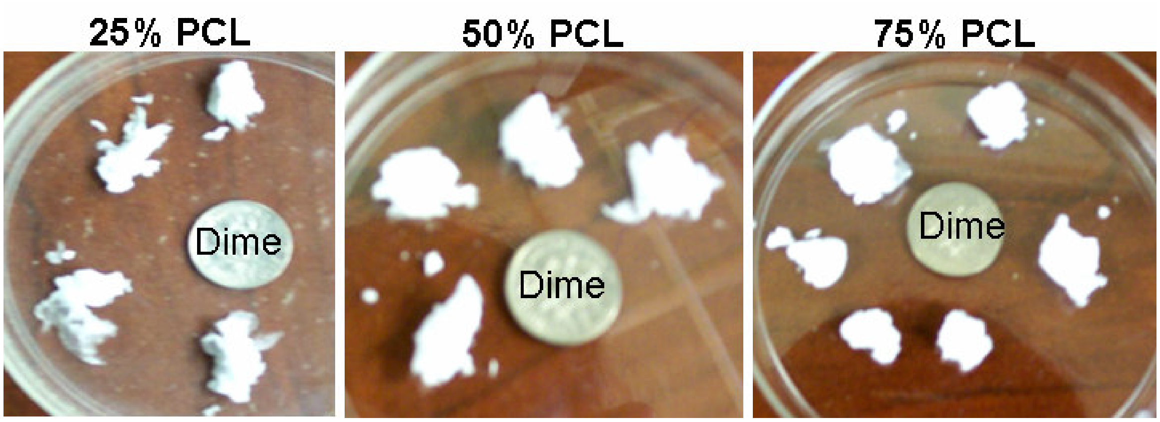

Figure 3.3. Influence of mixing chitosan and PCL in 25% acetic acid, on dimensional stability of scaffolds formed by freeze-drying. Solutions were frozen at -196°C and lyophilized at -80°C.

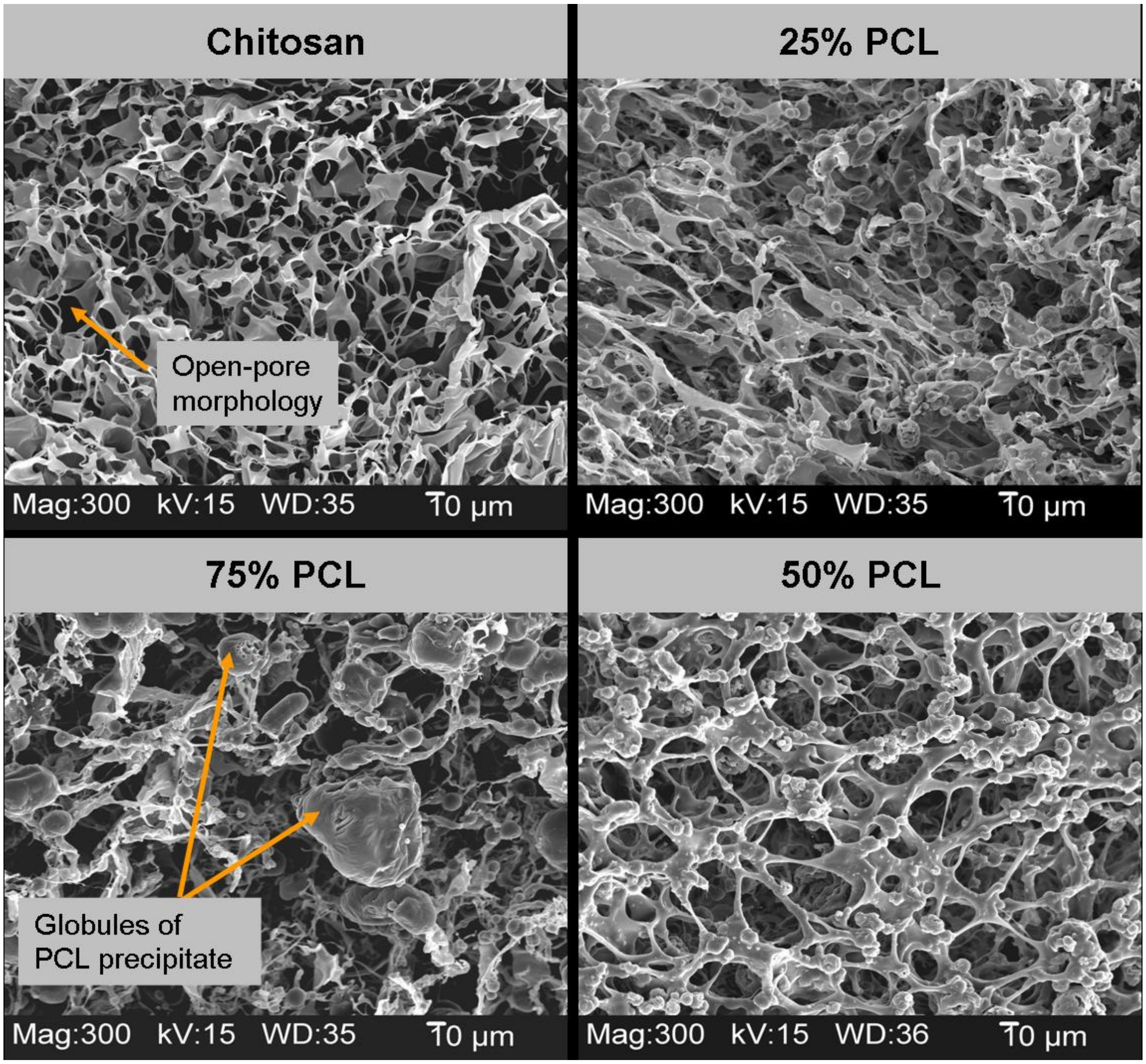

Figure 3.4. Influence of mixing chitosan and PCL in 25% acetic acid, on pore morphology of scaffolds formed by freeze-drying. Solutions were frozen at -196°C and lyophilized at -80°C.

Cross sectional view of chitosan scaffolds (**Figure 3.5A**) under SEM showed parallel layers without a regular pore structure. Top view of scaffolds showed a continuous layer with deposits. PCL appeared as spherical particles throughout the scaffold (**Figure 3.5B**), unlike fibers or the layer format of chitosan. This was observed in both top view and cross sectional views. The arrangement of PCL particles was random and agglomerated, without a definite pore structure.

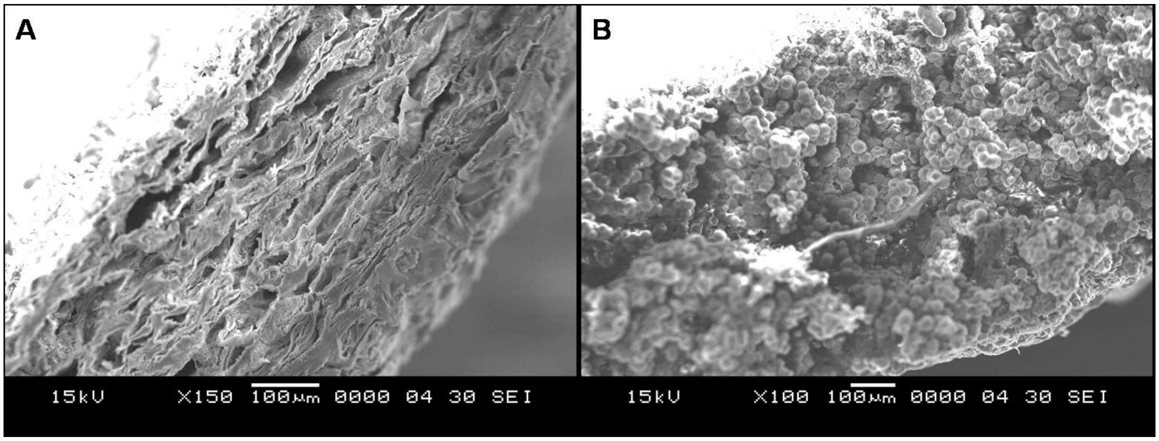

Figure 3.5. SEM images of scaffolds of A) Chitosan, obtained by freeze-gelation, and B) PCL, obtained by freeze-extraction.

3.4. DISCUSSION

Chitosan is typically dissolved in 0.2M- 0.5M acetic acid as it is insoluble in glacial acetic acid or in halogenated solvents used for dissolving PCL. On the other hand, PCL is soluble in glacial acetic acid and most organic solvents but not in water. Since acetic acid is completely miscible in water, an approach taken to combine the two polymers was to mix separate solutions of chitosan in 0.5M acetic acid and PCL in glacial acetic acid.

Mixing 3mL of chitosan with 1mL of PCL caused precipitation of PCL, irrespective of the concentrations of polymers or the mass ratios in which they were mixed. In this mixture, the total concentration of acetic acid was 25%. Although a small amount of chloroform was added to dissolve the PCL precipitate, phase separation remained a problem at higher PCL compositions. The reason for PCL precipitation was suspected to be the complete solubility of acetic acid in water. When the 1mL of acetic acid dissolved in water, PCL remained undissolved and hence phase separated. Therefore, the acetic acid- water ratio was raised successively until phase-separation of PCL stopped occurring. This was achieved by mixing 10mL of PCL solution with 3mL of chitosan solution, in which the concentration of acetic acid was 77%. Stirring this mixture for two minutes and slight warming formed single-phase solutions as the polymers were i) completely dissolved and ii) in dilute concentrations. However, for higher concentrations of chitosan or PCL, one has to optimize the solvent composition, mixing time, and temperature.

Chitosan and PCL formed stable emulsions in equilibrium ternary mixtures of chloroform, acetic acid and water at room temperature, but membranes cast overnight at room temperature showed phase separation. This is suspected to be due to fluctuations in room temperature as even slight changes in the temperature could disturb the equilibrium of these ternary mixtures.

Porous scaffolds of chitosan/PCL mixtures formed via. controlled rate freeze-drying did not show the structural stability that chitosan (Madihally and Matthew 1999) or PCL (Kweon, Yoo et al. 2003) scaffolds have. This could be attributed to the phase behavior of acetic acid, which was present in large amounts in the mixtures. During the freeze-drying process, the underlying mechanism responsible for forming pores is phase-separation of water and solid polymer. Upon freezing, the phase-separated water forms ice crystals which undergo sublimation during lyophilization, leaving behind a porous network in the polymer phase. The nucleation, growth and sublimation kinetics of acetic acid crystals are probably very different from those of water. Hence, a compact porous network could not be obtained by controlled-rate freeze-drying of acetic acid-water solutions.

PCL scaffolds formed by freeze-extraction had excellent stability and strength. The strength of the scaffolds decreased as PCL concentration and MW was lowered. This implied that the PCL fibers formed a well-connected porous network within the discs and the strength of the network was influenced by the amount and strength of PCL fibers. However, stable scaffolds of chitosan/PCL mixtures could not be obtained by freeze-extraction process either. The reason for this could be that the acetic acid-water mixtures which were frozen at -80°C, thawed immediately upon contact with acetone which was maintained at -20°C, and instantly dissolved. This is supported by the fact that the mixtures were not originally freezable at -20°C, despite the freezing point of water being 0°C and that of acetic acid being 16°C. Increasing the concentration and MW of the polymers may give better results. Other methods such as 3D printing could be explored to make porous scaffolds of chitosan/PCL mixtures (Hutmacher, Sittinger et al. 2004). In 3D printing, free-form fabrication of viscous polymer solutions through a micro-needle is done in a spatially defined three dimensional pattern. This technique offers better accuracy and repeatability of pore structure of scaffolds, but is expensive.

3.5. CONCLUSION

Hydrophilic chitosan and hydrophobic PCL were successfully mixed in a unique 77% acetic acid- water mixture without using toxic common solvents or complex chemical modification. The mass ratio of chitosan and PCL in the mixtures was easily alterable and uniform membranes could be formed. Formulation of porous scaffolds from these mixtures remains a target to be achieved.

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CHAPTER IV

INFLUENCE OF MIXING ON MECHANICAL PROPERTIES OF CHITOSAN

4.1. INTRODUCTION

In tissue engineering, mechanical properties of the scaffold greatly influence the chances of the implant success (Hutmacher 2000). These properties are dictated not only by the biomaterial constituting the scaffold, but also by the architecture and pore morphology of the scaffold. Chitosan has been a much sought after material in tissue engineering scaffolds, membranes, and hydrogels. However, despite good biological properties and other advantages, chitosan has limited mechanical strength as it is a naturally derived polysaccharide. To overcome these limitations, chitosan has been combined with several synthetic polymers that could extend mechanical integrity to the matrix. For example, addition of poly(vinylalcohol) increased the tensile strength of dry chitosan membranes from 70 to 220MPa and their extension from 4 to 11% (Park, Jun et al. 2001).

In this project, chitosan was mixed with a synthetic polyester polycaprolactone (PCL) by different mass ratios. This was achieved by dissolution in a unique acetic acid-water mixture, as chitosan and PCL are hydrophilic and hydrophobic, respectively. PCL is tough, with tensile properties to the tune of 31MPa strength, and 940% extension (Ng, Teoh et al. 2000). Mixture solutions were fabricated into uniform two dimensional (2D) membranes under varying process conditions. The focus of this study is to evaluate the influence of mixing chitosan and PCL and their relative mass ratios, on the tensile properties of mixture membranes. Effects of processing the membranes at different conditions were tested for optimum conditions. In order to simulate physiological conditions, a custom built chamber with constant circulation of phosphate buffer saline (PBS), maintained at 37°C was used to test the membranes in a completely wet state. For the first time, the fatigue properties of chitosan membranes and the effect of mixing with PCL on the same were evaluated using cyclical loading on the specimens.

4.2. MATERIALS AND METHODS

Chitosan (85% DD, MWs 50-190kD and >310kD) and PCL (MW 80kD) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals used were of reagent grade.

4.2.1. Preparation of samples: Three milliliters of chitosan (MW>310kD) dissolved in 0.5M acetic acid was added to 10mL of PCL dissolved in glacial acetic acid, in a glass vial. The concentrations used were 1% chitosan with 0.1%, 0.3% and 0.9% of chitosan for 25% PCL, 50% PCL and 75% PCL mixtures, respectively. The mixtures were slightly warmed and stirred for two minutes to obtain homogenous solutions. Fifteen milliliters of solutions were poured into Teflon dishes of 50mm diameter and dried overnight by the following three methods to form membranes of 80-100μm thickness:

i) at 37°C under controlled humidity conditions.

ii) in a water bath maintained at 55°C inside a fume hood

iii) in an oven maintained at 55°C (unsaturated humidity state)

Further, membranes obtained from the third method were solvent annealed using chloroform. For this purpose, membranes were exposed to an enclosed environment saturated with chloroform vapors at room temperature for 20 seconds. The chloroform evaporated within 5 minutes of removing the membranes from the chamber.

For cyclical tensile testing, thicker membranes were made by doubling the concentrations of chitosan and PCL used in the mixtures, but keeping the volumes constant. Therefore, 3mL of 2% chitosan was mixed with 10mL of 0.2%, 0.6% and 1.8% PCL to form 25%, 50% and 75% PCL mixtures, respectively. In these mixtures, the corresponding total polymer concentrations were 0.62%, 0.92% and 1.85%. To test the effect of total polymer concentration on mechanical properties of mixtures, a factorial design of experiments was created as shown in **Table 4.1**. In this table, entries a, b and c signify concentrations currently being used. In order to test the effect of mixing and total polymer concentration (TPC), all three mixtures need to be made with a fixed TPC of either 0.62% or 0.92% or 1.85%. According to this table, solutions with an 'NF' (meaning 'not feasible) entry cannot be made due to high concentrations of chitosan, which are too viscous to handle. Therefore, entries'd' and 'e' were made and compared with 'a'. This was accomplished by mixing 3mL of 1.3% and 0.7% chitosan with 10mL of 0.4% and 0.6% PCL, respectively (**Table 4.1**).

4.2.2. Uniaxial tensile testing procedure: The tensile properties of membranes were measured at different temperature and hydration conditions using an INSTRON 5542 machine (INSTRON Inc., Canton, MA). For this purpose, membranes of uniform thickness were cut into rectangular strips of approximately 50 mm×7.5 mm size, inserted between the grips and pulled to break at a constant cross head speed of 10 mm/min. To measure the thickness of membranes, digital micrographs of the cross section were obtained at various locations through an inverted microscope (Nikon, TE2000U, Melville, NY) equipped with a CCD camera. These images were quantified for thickness using image analysis software Sigma Scan Pro (SPSS Science, Chicago, IL). At least 10 to 12 images were analyzed per sample.

Samples were tested either in dry state or in the wet state at or 37°C using a custom-built environmental chamber. For wet 25°C, membranes were neutralized in 1N NaOH for 15 min, rinsed thoroughly under tap water and immersed in PBS for 30 minutes prior to testing. PBS was prepared by dissolving 8.0g NaCl, 0.2g KCl, 0.2g KH_2PO_4, and 2.17g Na_2HPO_4-$7H_2O$ in 975mL of distilled water, and adjusting the pH to 7.4. For testing at wet 37°C, samples were kept immersed in PBS during testing (**See Appendix I**). The buffer was maintained at 37°C in a bath and continuously circulated through the chamber using a peristaltic pump, with an inlet pipe at the bottom and an outlet pipe at the top of the chamber.

Table 4.1. Factorial design of experiments to test the effect of total polymer concentration on mechanical properties of mixture membranes. NF: Not feasible

Mixture composition	Total polymer concentration		
	0.62%	0.92%	1.85%
25% PCL	a	NF	NF
50% PCL	d	b	NF
75% PCL	e	f	c

4.2.3. Cyclical tensile testing procedure: To analyze their fatigue properties, membranes were subjected to cyclical uniaxial tensile loading. This was done at wet 37°C conditions only, as described in previous section. Membranes were cut into 50mm x

7.5mm size strips, neutralized with 1N NaOH for 15 minutes, washed thoroughly with water and hydrated with PBS for another 15 minutes. A custom built chamber surrounding the grips with constant circulation of PBS maintained at 37°C provided constant hydrating conditions. Samples were strained repeatedly between two preset fluctuating loads for 10 cycles at a constant crosshead speed of 10mm/min and the hysteresis loop behavior and fatigue stress/strain loss were analyzed. The load limits were predetermined by the linear portion of the load-extension curves obtained previously under monotonic loading. These limits were 0.2N to 2N for chitosan (MW>310kD), 0.2N- 1N for mixtures and chitosan (MW 50-190kD) and 0.2N- 8N for PCL membranes.

4.2.4. Calculation of tensile properties: Using the associated software Merlin (INSTRON Inc), the stress at break, strain at break [$(L-L_0)/L_0$ where L_0 is the initial length of the sample and L is the sample length at break], were determined. The elastic modulus was calculated from the slope of the linear portion of the stress-strain curve.

4.2.5. Statistical analysis: All experiments were repeated three or more times with triplicate samples for each group. Significant differences between two groups were evaluated using a one way analysis of variance (ANOVA) with 95% confidence interval. When $P<0.05$, the differences were considered to be statistically significant.

4.3. RESULTS

4.3.1. Influence of MW and hydration on monotonic tensile properties of chitosan:

The membranes were first tested in the dry state. Results (**Table 4.2**) showed that the membranes were very brittle, exhibiting a break strain as low as 4-6%, very high stress at break (56- 88MPa), and an elastic modulus of 2.35-3.50GPa. The elastic modulus of chitosan decreased with increasing MW. Also, the tensile properties varied significantly with the cross-head speed used while testing; when a random crosshead speed of 210mm/min was used, the elongation at break value was nearly 50% lower than at 10mm/min speed (data not shown).

Table 4.2. Influence of molecular weight and hydration on tensile properties of chitosan membranes.

Property	**Hydration**	**Molecular weight**		
		50- 190kD	**190- 310kD**	**>310kD**
Break stress (MPa)	Dry	58.47 ± 2.16	78.32 ± 7.04*	84.87 ± 3.84*
	Wet 25^{0}C	2.50 ± 0.39**	2.46 ± 1.58	1.3 ± 0.49**
	Wet 37^{0}C	1.72 ± 0.71	1.58 ± 1.39	1.04 ± 0.10
Break strain (%)	Dry	4.46 ± 1.81	12.97 ± 9.74	6.30 ± 1.92
	Wet 25^{0}C	44.38 ± 13.63	54.55 ± 15.79	60.35 ± 6.09
	Wet 37^{0}C	36.85 ± 6.82	67.16 ± 16.67	59.54 ± 4.8
Elastic Modulus (MPa)	Dry	2536.23 ± 156.75	3177.2 ± 412.71	3411.28± 288.24
	Wet 25^{0}C	6.47 ± 0.78***	5.40 ± 1.45***	2.49 ± 0.49
	Wet 37^{0}C	5.43 ± 0.79	5.12 ± 0.99	2.04 ± 0.72

* P< 0.01 with respect to break stress of 50-190kD group in dry state
** P<0.01 between the two indicated groups.
*** P< 0.01 with respect to e-modulus of >310kD in wet 25^{0}C state.

Next, chitosan membranes were tested in a wet state at 25°C. These results showed a significant difference in the stress-strain behavior relative to dry samples (**Figure 4.1**). The break stress of dry samples was lowered by an order-of-magnitude whereas samples endured a longer elongation (45-60%) at break in the wet state. With increase in MW, membranes exhibited greater strain but lesser stress at break and a lower elastic modulus (**Table 4.2**). When samples were tested at 37°C, no significant differences were observed in the measured parameters relative to 25°C (**Figure 4.1** and **Table 4.2**). Overall, increasing the MW decreased the elastic modulus with >310kD chitosan showing the lowest values.

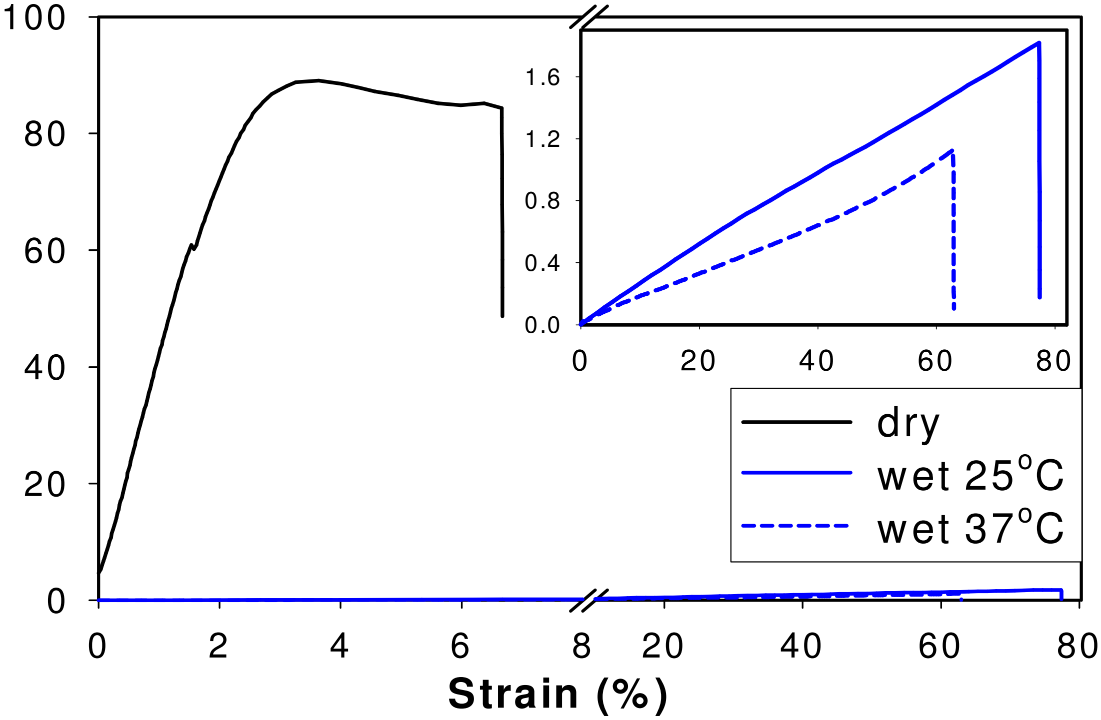

Figure 4.1. Stress-strain behavior of chitosan membranes tested in dry and different hydrating conditions under monotonic tensile loading.

4.3.2. Influence of mixing and processing conditions on monotonic tensile properties of chitosan: PCL membranes cast after dissolving in chloroform have a break stress of 10 MPa and an elongation at break of 400-1000% (**Figure 4.2**). It is desired to obtain such high values for the mixture membranes. The stress-strain behavior of mixture membranes formed at 25°C or at 37°C showed characteristic curves between chitosan and PCL (formed from chloroform) membranes (**Figure 4.2**). However, these results were independent of composition, the break stress and strain of mixtures being closer to those of chitosan (**Table 4.2**). These values decreased with increased PCL composition. Mixture membranes formed by drying at 55°C in oven showed break stress and strain (**Figure 4.3**) slightly higher than those obtained from the water-bath or that of chitosan (**Table 4.2**). Annealing these membranes with chloroform vapors increased the tensile stress and strain by at least 100% relative to oven-dried membranes (**Figure 4.3**). Elastic modulus of 75% PCL was significantly higher than other mixtures or chitosan alone.

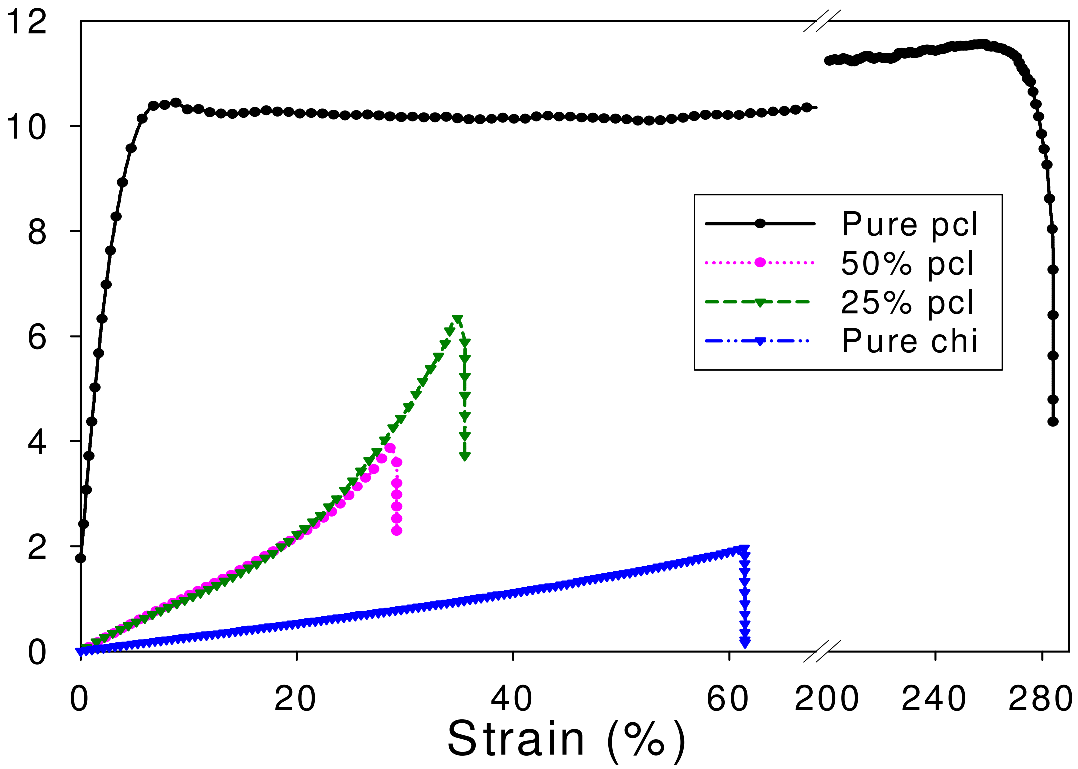

Figure 4.2. Stress-strain behavior of pure PCL and chitosan membranes in comparison to their mixture membranes, tested at wet 37°C under monotonic tensile loading.

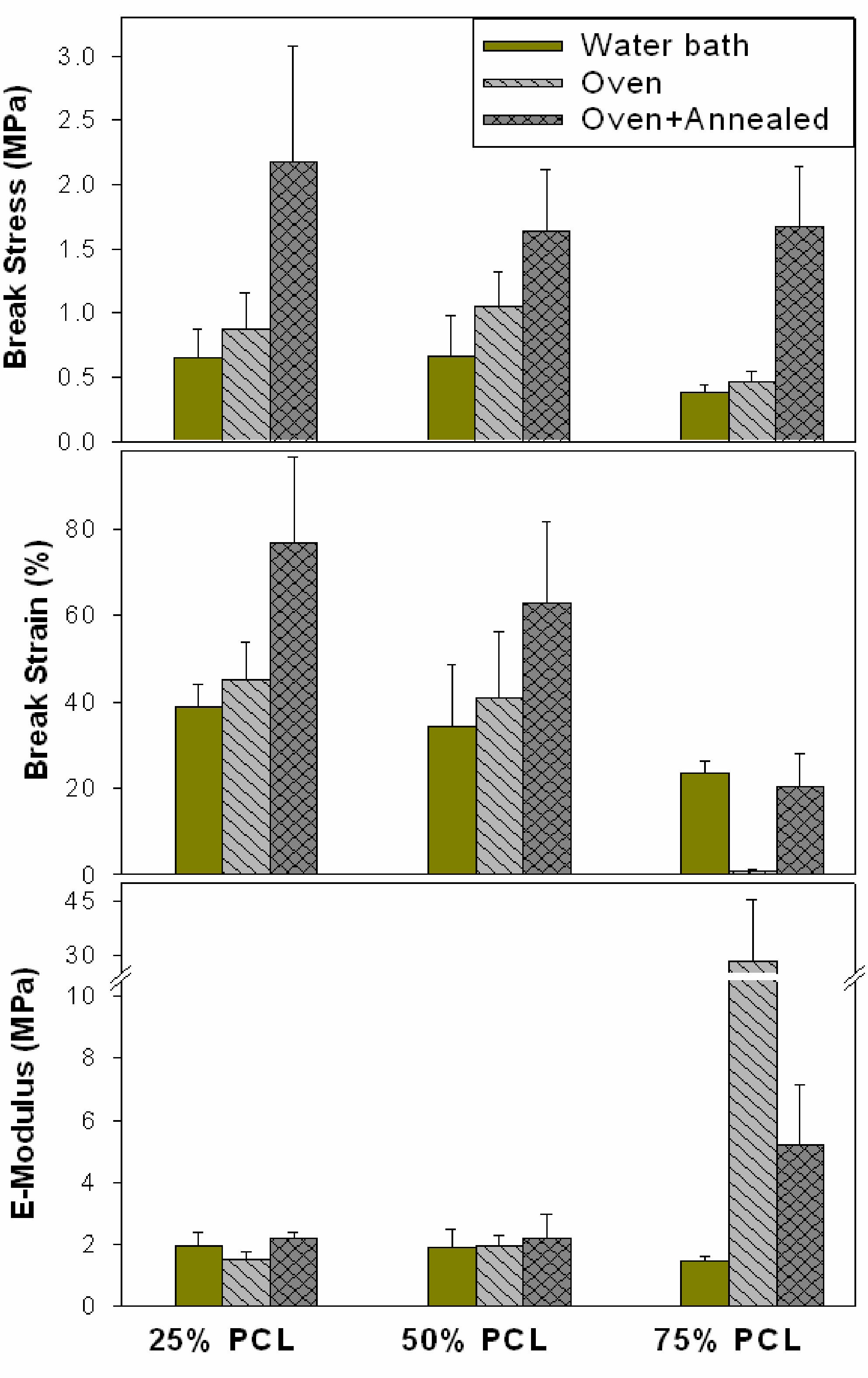

Figure 4.3. Influence of composition and drying conditions on tensile properties of chitosan-PCL mixture membranes, tested at wet 25°C conditions.

4.3.3. Influence of MW on fatigue properties of chitosan under tensile loading: First, when high MW chitosan membranes were subjected to fluctuating loads 0.2N-2N for 10 cycles, they did not break (**Figure 4.4**). Maximum deformation occurred in the first cycle and there was minimal deformation in subsequent cycles indicating no additional strain storage in the samples after the first cycle. Similarly, low MW chitosan membranes which were subjected to 10 cycles of loads 0.2N-1N, also withstood the stresses without breaking.

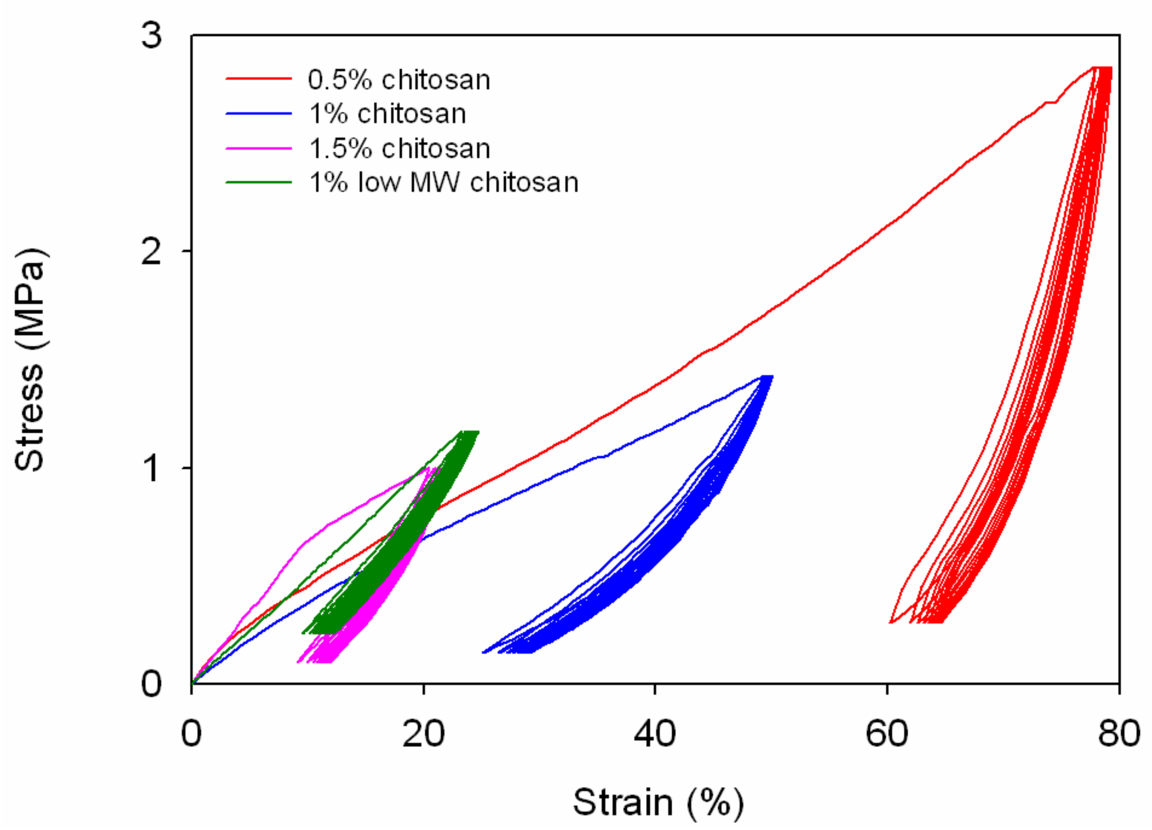

Figure 4.4. Influence of molecular weight and concentration on fatigue properties of chitosan membranes under cyclical tensile loading, when tested at wet 37°C conditions.

4.3.4. Influence of mixing on fatigue properties of chitosan under tensile loading:

Similar to chitosan, mixture membranes of (variable total concentrations) which were subjected to 10 cycles of loads 0.2N-1N also withstood the stresses without breaking **(Figure 4.5)**. PCL did not yield or elongate significantly within the specified load limits. However, mixture membranes exhibited at least 10% higher strain than chitosan despite lower load limits and the strain values increased with increased PCL content. Among the mixtures, 50% PCL membrane showed maximum strain.

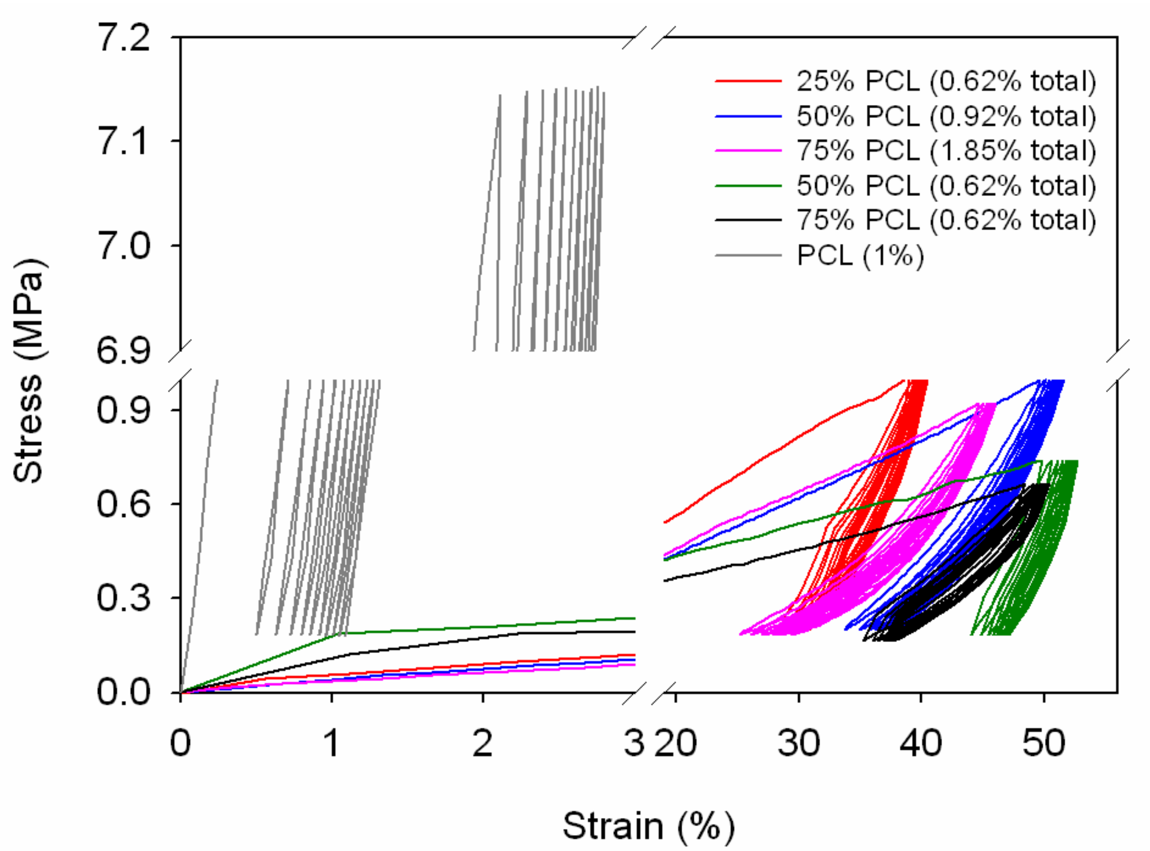

Figure 4.5. Influence of composition and total polymer concentration on fatigue properties of chitosan-PCL mixture membranes under cyclical tensile loading, when tested at wet 37°C conditions.

4.3.5. Influence of total polymer concentration on tensile properties of chitosan-PCL mixtures: Chitosan membranes of 0.5%, 1% and 1.5% concentration sustained 10 cycles of load without break (**Figure 4.4**). As concentration increased, membranes were subjected to lower stress and achieved lower extension due to increase in the thickness of membranes with concentration. Similar to chitosan, mixture membranes of TPC 0.62% sustained 10 cycles of load without break **(Figure 4.5)**. Higher strain was exhibited by 50% PCL compared to 25% or 75% PCL, similar to mixtures with variable TPC. It maybe noticed that the initial strain of 50% PCL and 75% PCL membranes increased as the total concentration decreased from 0.92% and 1.85% respectively to 0.62%.

4.4. DISCUSSION

PCL membranes cast after dissolving in chloroform showed a break stress of 10MPa (**Figure 4.2**) and an elongation at break of 400-1000%. The objective is to imbibe these properties by incorporating PCL in chitosan membranes. Chitosan/PCL composite membranes formed by drying at 25°C or at 37°C were not uniform, and they randomly separated into smooth and rough phases. The break stress and strain values decreased with the increased PCL composition, probably due to discontinuity of chitosan phase at higher PCL content. This suggested a need for better drying process that could minimize separation of two polymers.

Forming the membranes at 55°C, especially in an oven showed higher stress and strain at break, compared to membranes dried at 25°C or at 37°C or pure chitosan. However, these properties were independent of composition, probably due to a) crystal structure of PCL in acetic acid, or b) low MW of PCL relative to chitosan, which resulted in incomplete inter-connectivity of PCL. This suggested a need for further processing of the membranes. Since PCL crystal structure (or ductility) is dependent on the solvent (chloroform supporting more ductility than acetic acid), one way to improve the ductility of the mixture membranes is to relax PCL and increase its inter-connectivity using chloroform. Higher stress, strain, and modulus values were obtained for the mixtures by annealing with chloroform vapors, which could be attributed to better inter-connectivity of PCL. At low PCL content, the decreased inter-connectivity could be due to the low MW of PCL relative to chitosan. Therefore, the effect of MW ratios of PCL and chitosan needs further investigation. Others have co-dissolved of chitosan and hydrophilic polymers such as PVOH (Srinivasa, Ramesh et al. 2003), PEO (Jin, Song et al. 2004), PEG (Suyatma, Tighzert et al. 2005), and PVP (Qurashi, Blair et al. 1992). Mechanical properties of these mixtures tested mostly in the dry state, showed that the tensile strength of chitosan was reduced, and the elongation was increased.

Unlike monotonic loading, effect of MW of chitosan was not significant under cyclical loading in the tested load range. All materials showed maximum deformation in the first cycle, suggesting that they could endure many more cycles. However, the tested membranes had changes in mass ratios of two polymers as well as the total concentration. Since the packing density of polymers varies with total concentration, it may have a significant influence on mechanical properties. Therefore, mixture membranes of varying PCL composition were made with a fixed total polymer concentration of 0.62% and tested. Results indicated that varying total concentration altered only the monotonic tensile properties and did not significantly influence the fatigue properties (hysteresis behavior). The absolute values of stress and strain values decreased due to lower polymer concentration but the trend ‘among’ the mixtures was similar to that obtained with varying total polymer concentrations. These results suggest that in mixtures with current volume ratio, the relative amount of PCL has a major impact on the tensile properties. Hence, it may be concluded that at any total concentration, the mass ratios of chitosan and PCL have a significant influence on mechanical behavior.

Further analysis is necessary to understand the influence of a broader range of total polymer concentration on other aspects of fatigue properties such as energy loss per hysteresis loop and strain per cycle. In addition, the analysis should be extended to determine the fatigue life (number of cycles of fluctuating stress and strain endured before permanent structural change) and fatigue limit (the maximum fluctuating stress endured for infinite cycles) of these polymers. Such information would help streamline these polymers for different biomedical applications.

4.5. CONCLUSION

Tensile properties of mixture membranes were comparable to that of chitosan. Importantly, these properties were alterable depending on the mixture composition and drying temperature. All pure polymer and mixture membranes were able to sustain cyclical loading within load limits corresponding to their elastic region.

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CHAPTER V

INFLUENCE OF MIXING CHITOSAN AND PCL ON *IN VITRO* DEGRADATION PROPERTIES

5.1. INTRODUCTION

In tissue engineering, the degradation or resorption rate of a scaffold has to match the rate of tissue formation. Faster degradation is not desirable as the growing tissue will remain unsupported, resulting in collapse. At the same time, slower resorption rate might cause an unwanted immune reaction or host response. Therefore, a trade-off has to be achieved between structural integrity and rate of resorption. Also of importance are the products of degradation, as they have to be biocompatible.

Chitosan and PCL are two biocompatible and biodegradable polymers, which are being explored in tissue engineering matrices. However, both these polymers are known to degrade slowly, by different mechanisms. Chitosan degrades into oligosaccharides by enzymatic action of lysozyme present in the body (maximum at pH 4.5-5.5) (Davies, Neuberge.A et al. 1969; Shigemasa, Saito et al. 1994), whereas PCL undergoes non-enzymatic bulk hydrolysis of its ester linkage, followed by fragmentation into oligomers (Pitt, Chasalow et al. 1981). Chitosan takes 2-3 months to degrade and is a function of molecular weight (MW), degree of deacetylation (DD) and local pH. This rate could be accelerated by introducing synthetic polymers such as PLGA that produce acidic byproducts which reduce local pH (Moshfeghian 2005).

The degradation rate of PCL is lower than chitosan, taking anywhere from 6 months to 1 year (Wise 1995). This could be partially attributed to its hydrophobic nature, which prevents water absorption and permeability. The degradation rate of synthetic polymers could be increased by adding a natural polymer, as it may result in reduced crystallinity and hydrophobicity (Bastioli, Cerutti et al. 1995; Wu 2005).

In this project, chitosan was mixed with PCL in different mass ratios to obtain a composite with improved biomechanical properties. Mixing was done by dissolution in a unique acetic acid-water mixture. The focus of this study was to investigate the effects of chitosan/PCL composites and the acidic solvents on the rate of biodegradation *in vitro*. Degradation rate was characterized by weight loss of membranes and changes in pH of media.

5.2. MATERIALS AND METHODS

Chitosan (~85% DD, MW >310kD), PCL (MW 80kD) and hen egg white (HEW) lysozyme (46400 U/mg) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals used were of reagent grade.

5.2.1. Preparation of samples: Three milliliters of chitosan dissolved in 0.5M acetic acid was added to 10mL of PCL dissolved in glacial acetic acid, in a glass vial. The concentrations used were 2% chitosan with 0.2%, 0.6% and 1.8% of PCL for 25% PCL, 50% PCL and 75% PCL mixtures, respectively. The mixtures were slightly warmed and stirred for 2 minutes to obtain homogenous solutions. These solutions were cast in Teflon dishes for 24 hours in an oven at 55°C to form uniform membranes of 100-120μm thickness.

5.2.2. Evaluation of *in vitro* degradation kinetics: These studies were conducted for a period of four weeks, as reported previously by our lab (Huang, Onyeri et al. 2005). Chitosan, 50% PCL and pure PCL membranes were cut into 20mm×20mm size strips and their initial weights were measured. The strips were neutralized in 1N NaOH, washed with deionized water, sterilized in absolute alcohol for 30 minutes and washed thoroughly in sterile PBS (prepared as described in section 4.2.2, and autoclaved in wet cycle at 121°C for 20 minutes), prior to incubating in 10mL PBS with and without 100mg/L HEW lysozyme. Samples without lysozyme were taken as control. Incubations were carried out in 20mL vials with a ~15mm diameter hole drilled in the caps and covered on the inside with 0.22μm filters. Media was replaced every two days.

At two day intervals, three samples per group were sacrificed for degradation analysis and incubation media was collected for pH analysis. Sacrificed samples were washed with deionized water, dehydrated using absolute alcohol and dried to constant weight in a vacuum desiccator at ambient conditions prior to final weight determination. Digital images were also obtained to characterize dimensional changes. Samples sacrificed after thirty minutes of incubation were considered as day zero samples.

5.2.3. Statistical analysis: All experiments were repeated three or more times with triplicate samples for each group. Significant differences between two groups were evaluated using a one way analysis of variance (ANOVA) with 95% confidence interval. When $P<0.05$, the differences were considered to be statistically significant.

5.3. RESULTS

PCL membranes negligible showed weight loss over the period of study (**Figure 5.1**). Chitosan membranes showed maximum decrease in weight (nearly 20%) within the first

30 minutes (day 0), and did not degrade much over the remaining study period. Similar to chitosan, 50% PCL showed significant weight loss (15%) within the first 30 minutes itself, indicating that the weight loss could be due to loss of chitosan. The effect of lysozyme on degradation was not significant in any group, despite using a hundred fold concentration, compared to natural levels. Interestingly dimensional changes in all membranes were negligible (hence, data not shown), irrespective of composition or presence of lysozyme.

The solubility and lysozyme-mediated degradation of chitosan are known to be highly dependent on pH of the medium. Chitosan is soluble in water only at pH<6.2, while the degradation rate is maximum at pH 5.2. Hence, the pH of incubation media was analyzed. Data suggest a decrease from the initial value of 7.4 in all groups (**Figure 5.1**). At any measured time point, pH of media containing PCL was the lowest, followed by 50% PCL and chitosan, although there was no significant difference between the two groups. As there was negligible degradation in all samples after day zero, the drop in pH is attributed to the solubility of carbon dioxide in water, to form carbonic acid. The atmosphere has 0.03% CO_2 which can reduce the pH of water from 7 to 5.5, depending on several factors including temperature and pressure (Lenntech). In this experiment, the pH drop could be higher due to the presence of 5% CO_2 in the incubator. Therefore, a stronger buffer is required for degradation experiments.

Interestingly, on day zero, there was a marginal increase in the pH of chitosan and 50% PCL sample media, from 7.4 to 7.5, despite thorough washing of NaOH neutralized membranes. This could be due to semi-stable adherence of NaOH, selectively to chitosan.

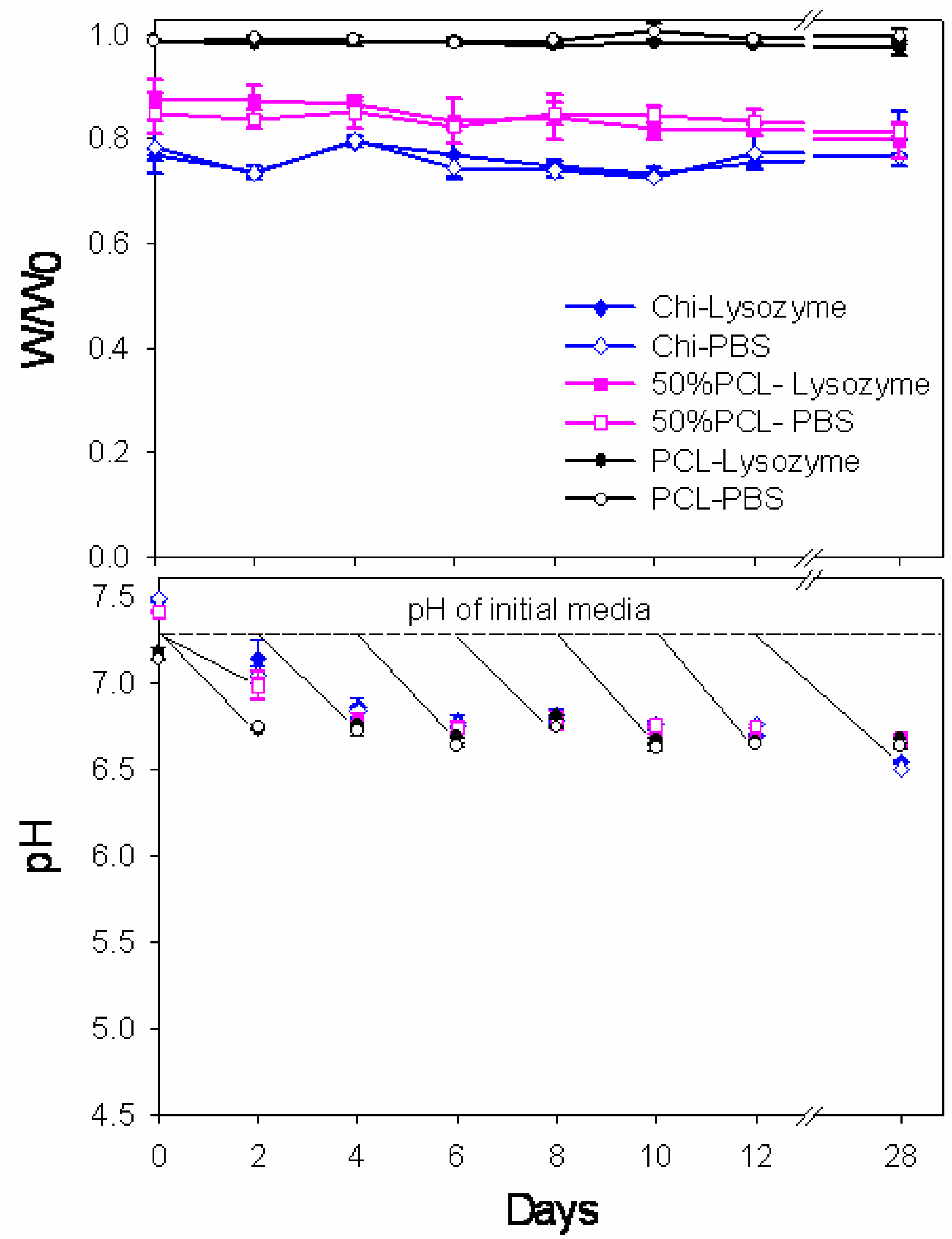

Figure 5.1. Influence of mixing with PCL on *in vitro* degradation properties of chitosan membranes- transient changes in weight loss and pH. Membranes were incubated in PBS containing hen egg white lysozyme (100mg/L) under sterile conditions at 37°C.

5.4. DISCUSSION

The degradation rate of chitosan was low despite very high concentration of lysozyme used in this study, relative to physiological levels. This is attributed to the 85% DD of chitosan used in this study, as previous research has shown that highly deacetylated chitosan is less susceptible to lysozyme (Pangburn, Trescony et al. 1982). Lysozyme had no effect on degradation of chitosan probably due to the pH conditions. The optimum pH for lysozymal degradation of chitosan is 5.2 (Nordtveit, Varum et al. 1996), whereas the pH was at an average of 6.8 throughout the experiment.

PCL also showed negligible degradation, probably due to the MW. High MW PCL degrades slowly until the first stage of random hydrolytic chain scission, which reduces the MW to 15kD or less (Cha and Pitt 1990). The initial weight loss of mixtures was attributed to chitosan only, suggesting that there was no interaction between chitosan and PCL between the mixtures.

The degradation rate of chitosan-PCL mixtures should be accelerated by changing the individual degradation rates as well as the matrix architecture. This can be done by using chitosan and PCL of MW~10kD. The MW of chitosan can be easily manipulated by depolymerization reaction using nitrous acid which involves the nitrosation of amine groups and cleavage of β-glycosidic linkages of polymeric chain (Allan and Peyron 1995). However, lowering the MW compromises the mechanical strength of these polymers. Therefore, these two requirements must be balanced, in order to choose an optimum MW. Degradation rate could also be accelerated by lowering the total polymer composition in the mixtures. In this study, mixtures were formed with nearly 4:1 chain length ratio of chitosan and PCL. Inverting this ratio would result in an altered packing assembly of chitosan and PCL fibers with the PCL chains forming the backbone. Such an orientation might make the chitosan molecules shorter and more accessible to lysozyme molecules resulting in accelerated degradation.

5.5. CONCLUSION

The degradation rates of chitosan or PCL were not significantly altered in the blends. The effect of lysozyme was also negligible. The MWs chitosan and PCL should be lowered for faster degradation.

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CHAPTER VI

INFLUENCE OF MIXING CHITOSAN AND PCL ON BIOREGULATORY PROPERTIES

6.1. INTRODUCTION

Chitosan has hence been a subject of many investigations in tissue engineering (Chupa, Foster et al. 2000; Lahiji, Sohrabi et al. 2000; Chung, Yang et al. 2002; Zhu, Ji et al. 2002; Mizuno, Yamamura et al. 2003) as it has shown support for biological activity of diverse cell types, despite lacking specific cell binding domains. Chitosan can complex with other uncharged or anionic polymers, and bind with negatively charged proteins due to positive charge and reactive functional groups that help regulate cellular and antibacterial activity. In order to enhance the physicomechanical properties of chitosan based matrices, chitosan was combined with PCL in this project, using a unique concoction of acetic acid and water. PCL has limited abilities to elicit controlled cellular responses.

The focus of this study is to evaluate the cytocompatibility of formed chitosan/PCL mixtures, and of the strong acids that were used as solvents. Chitosan and PCL have opposite nature in terms of water affinity and charge density. Chitosan is hydrophilic with a positive charge density, whereas PCL is an uncharged hydrophobic polymer. Therefore, chitosan and PCL mixtures represent a model system for studying the influence of charge density and water absorption on cellular activity. In addition, both chitosan and PCL lack cell binding domains. So their mixtures present an opportunity to investigate non-receptor mediated adhesion, if any.

Chicken chorioallantoic membrane (CAM) assay is a simple and reliable platform to study cytotoxicity and angiogenesis (the formation of new blood vessels from existing vasculature) *in situ* (Ribatti, Nico et al. 2001). CAM is a double layer of mesoderm on the outer side of an early embryo, extremely rich in vascular network which connects to the embryonic circulation by the allantoic arteries and veins. The physicochemical properties of implanted biomaterials regulate the structure and function of microvasculature (Sieminski and Gooch 2000). CAMs are wounded and biomaterials are placed on top to evaluate the cytocompatibility, and ability to coordinate wound closure, and vascular integration.

In this study, the influence of chitosan-PCL mixture membranes on cellular viability, proliferation, spreading, and cytoskeletal morphology was evaluated. Additionally, cytotoxicity of these mixtures in 3D scaffold form and support to angiogenesis was evaluated *in situ* by CAM assay.

6.2. MATERIALS AND METHODS

Chitosan (~85% DD, MW >310kD) and PCL (MW 80kD) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals used were of reagent grade. Mouse embryonic fibroblasts (MEFs, STO cell line) were purchased from American Type Culture Collection (Walkersville, MD).

6.2.1. Preparation of samples: Sterile chitosan solutions were prepared by suspending the chitosan in water and autoclaving at 121°C in a wet cycle for 20 minutes. Acetic acid equivalent to 0.5M was added in a sterile laminar flow hood to dissolve the chitosan. Three milliliters of chitosan was added to 10mL of PCL dissolved in glacial acetic acid in a glass vial. The concentrations used were 1% chitosan with 0.1%, 0.3% and 0.9% of PCL for 25% PCL, 50% PCL and 75% PCL mixtures, respectively. The mixtures were slightly warmed and stirred for two minutes to obtain homogenous solutions. These solutions were poured into 24-well tissue culture plates, 250µL each and air-dried overnight in the oven at 55°C (third method of membrane formation). Tissue culture plastic surface was used as control. Formed membranes were neutralized with 1N NaOH for 30 minutes, washed twice with sterile phosphate buffer saline (PBS) for 5 minutes each time, and stored in PBS until ready for cell seeding.

6.2.2. Cell culture and seeding: Mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 4mM L-glutamine, 4.5 g/L sodium bicarbonate, 0.1mM β-mercaptoethanol, 100U/mL penicillin-streptomycin, 2.5µg/mL amphotericin and 10% fetal bovine serum (FBS). The cultures were maintained in 5% CO_2 at 37°C, with medium changes every 48 hours. Polymer coated tissue culture well-plates were seeded with 10000cells/well and incubated. Media was replaced every 2 days.

6.2.3. Evaluation of cell viability: After two days, cellular viability was tested using MTT-formazan assay, as reported previously (Chupa, Foster et al. 2000). The medium was replaced with 0.5 mL of MTT solution (2 mg/mL in PBS) (Sigma Chemical Co., Saint Louis, MO) followed by incubation for two hours at 37°C. Then MTT solution was replaced with dimethyl sulfoxide (DMSO) to dissolve formazan and the absorbance of the solution was measured at 540nm.

6.2.4. Evaluation of cell spreading: Morphological changes were monitored at regular intervals using an inverted microscope (Nikon, TE2000U, Melville, NY). Digital micrographs were captured from representative locations and assessed using an image analysis software (Sigma Scan Pro, SPSS Science, Chicago, IL) for cell spreading and shape factors (shape factor is defined as $4\,\pi \times \text{area}/\text{perimeter}^2$; when the number is closer to 1, the cell shape is closer to a circle).

6.2.5. Evaluation of cytoskeletal organization: For cytoskeletal actin staining, cells were first fixed in 3.7% formaldehyde for 30min, washed with PBS, permeabilized with 100% ethanol (kept at -20°C) at 4°C for 60min and incubated with Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR) in the dark at 4°C for 30 minutes. Actin distribution in cells was observed under fluorescence microscopy and digital images were obtained using a CCD camera.

6.2.6. Evaluation of cytotoxicity by chorioallantoic membrane assay: Fertilized white leghorn eggs (Truslow Farms, Chestertown, MD) were incubated at 37° C and 70% relative humidity for three days upon receipt. On the morning of the fourth day, the eggs were placed horizontally in incubation trays with the rocker turned off for four to six hours. The eggs were gently cracked into 100 x 20 mm petri dishes and incubated at 37.2° C, 95% relative humidity, and 5% CO_2 for 24 hours.

A small vannas scissors were used to create a small hole in the surface of the chorioallantoic membranes (CAMs), away from major vessels and not leading directly to the embryo's ventral side **(Figure 6.1)**. CAMs were allowed to bleed but not hemorrhage. Sections of 2mm size were cut from chitosan, PCL and the mixture scaffolds obtained by freeze-drying solutions in 25% acetic acid (**Chapter 3, section**

3.2.1). The wound was covered with these scaffold sections. The biomaterials were allowed to sit on the wound and were observed 48 hours after the wound had been made. Wounded CAMs without any biomaterials were taken as control. Photographs were obtained using a Quantum Digital Imaging camera attached to a WILD microscope.

6.2.7. Statistical analysis: All experiments were repeated three or more times with quadruplicate samples. Obtained values in each experiment were normalized with the control samples. Significant differences between two groups were evaluated using a one way analysis of variance (ANOVA) with 95% confidence interval. When $P<0.01$, the differences were considered to be statistically significant. The distributions of cell spreading area were plotted as box plots, to show the 10th, 25th, 50th, 75th and 90th percentiles. Values outside 95th and 5th percentiles were treated as outliers.

6.3. RESULTS

6.3.1. Influence of mixing on cell viability: MEF activity was evaluated on oven-dried membranes formed inside 24-well plates. Mixture membranes supported cell adhesion and spreading, similar to chitosan membranes (**Figure 6.2**). Therefore the polymers and solvents used in these mixtures did not cause any cytotoxicity and are biocompatible. After 2 days, a significant increase in viability was observed on 50% and 75% PCL mixtures relative to tissue culture plastic or chitosan (**Figure 6.3**). Similar trend was observed when culture duration was extended to 5 days with increase in the absorbance.

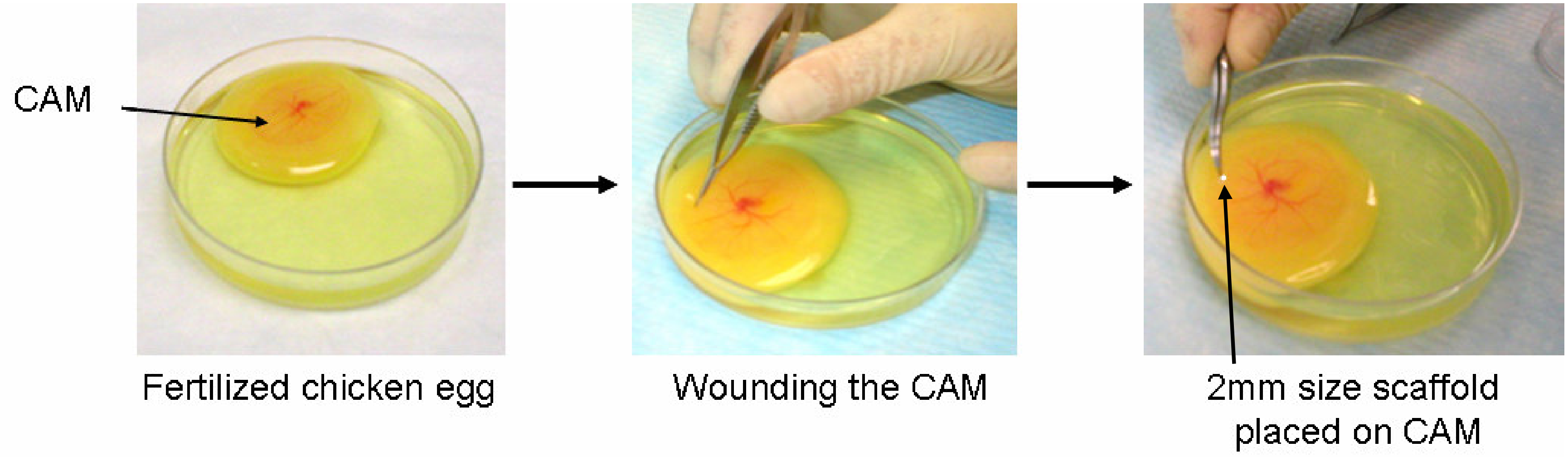

Figure 6.1. Chicken chorioallantoic membrane (CAM) assay to study cytotoxicity of chitosan/PCL scaffolds.

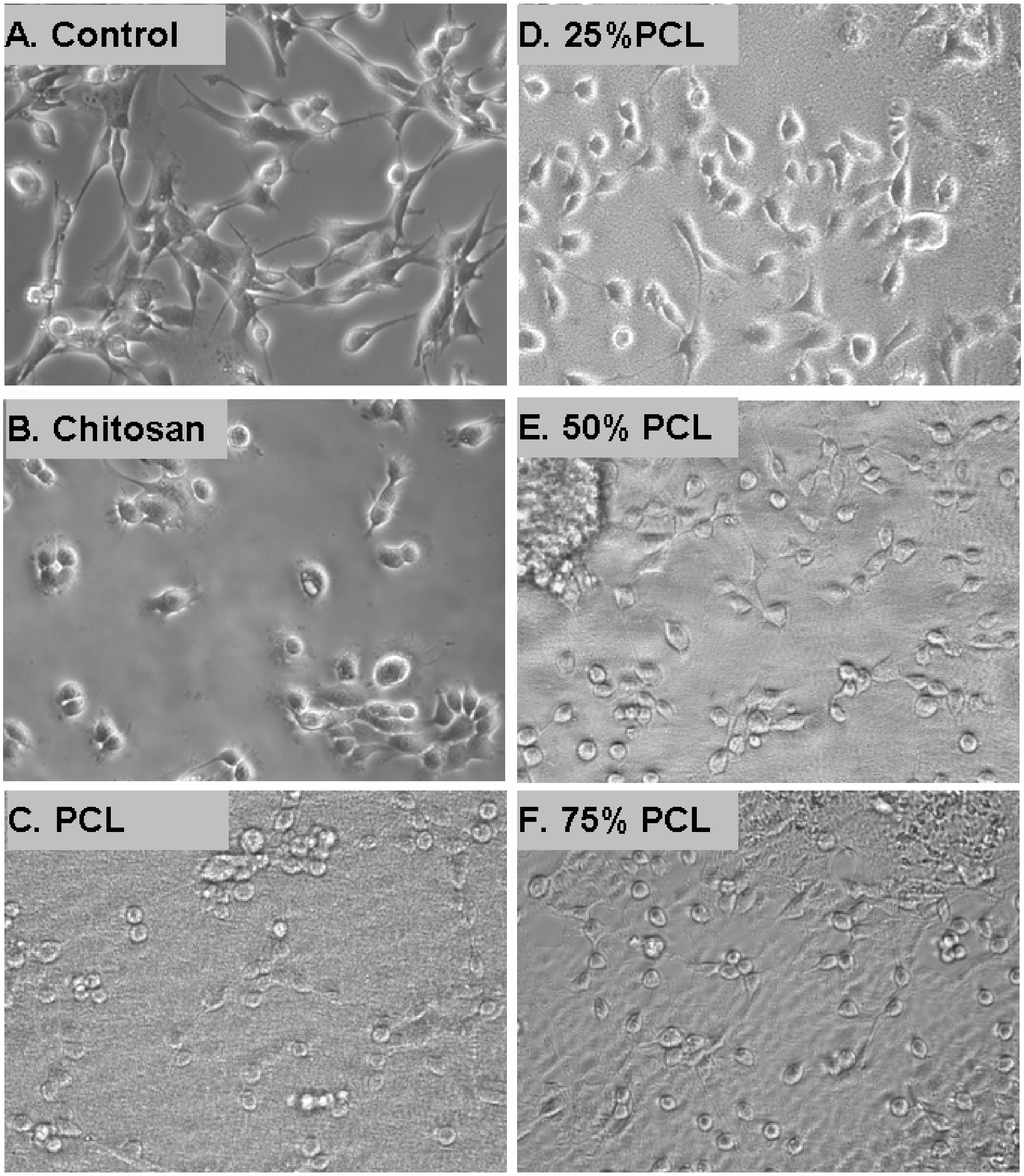

Figure 6.2. Influence of PCL mass ratio on shape and spreading of mouse embryonic fibroblasts on chitosan-PCL mixture membranes. Phase-contrast micrographs after 48 hours.

6.3.2. Influence of mixing on cell spreading and shape: All chitosan surfaces showed a shape factor of ~0.72±0.1, rather than the extended ~0.4±0.1 spindle shapes on tissue culture plastic. With the increase in PCL content, the shape factor steadily decreased to 0.43±0.16 approaching the tissue culture plastic. The cell spreading area analysis showed a significant decrease in the spreading area from control to chitosan (**Figure 6.3**), which is consistent with the shape factor results. There was no significant difference in the spreading area among the mixture membranes although these values were higher than on chitosan membranes (**Figure 6.3**). Pure PCL showed higher cell-spreading area compared to all the other membranes.

6.3.3. Influence of mixing on cytoskeletal organization: Actin staining images showed a significant alteration in the cell shape on chitosan membranes with the absence of typical spindle shape of MEFs (**Figure 6.4**). In addition, significant reduction and reorganization in the actin fibers were observed on chitosan and PCL membranes where actin was localized in the perinuclear space of the cell rather than cortical region. On tissue culture plastic, actin fibers were present in the entire intracellular compartment. However, mixture membranes showed peripheral distribution of actin filaments resembling more of tissue culture plastic, despite a reduction in cell spreading area.

6.3.4. Influence of mixing on cytotoxicity and vasculature support: CAMs were wounded in an effort to stimulate vessel growth and to provide a surface for biomaterial attachment. On control group CAMs, blood clotting took place at the wound site. Vascular network was not formed and the embryo died before 48hours. On wounds with

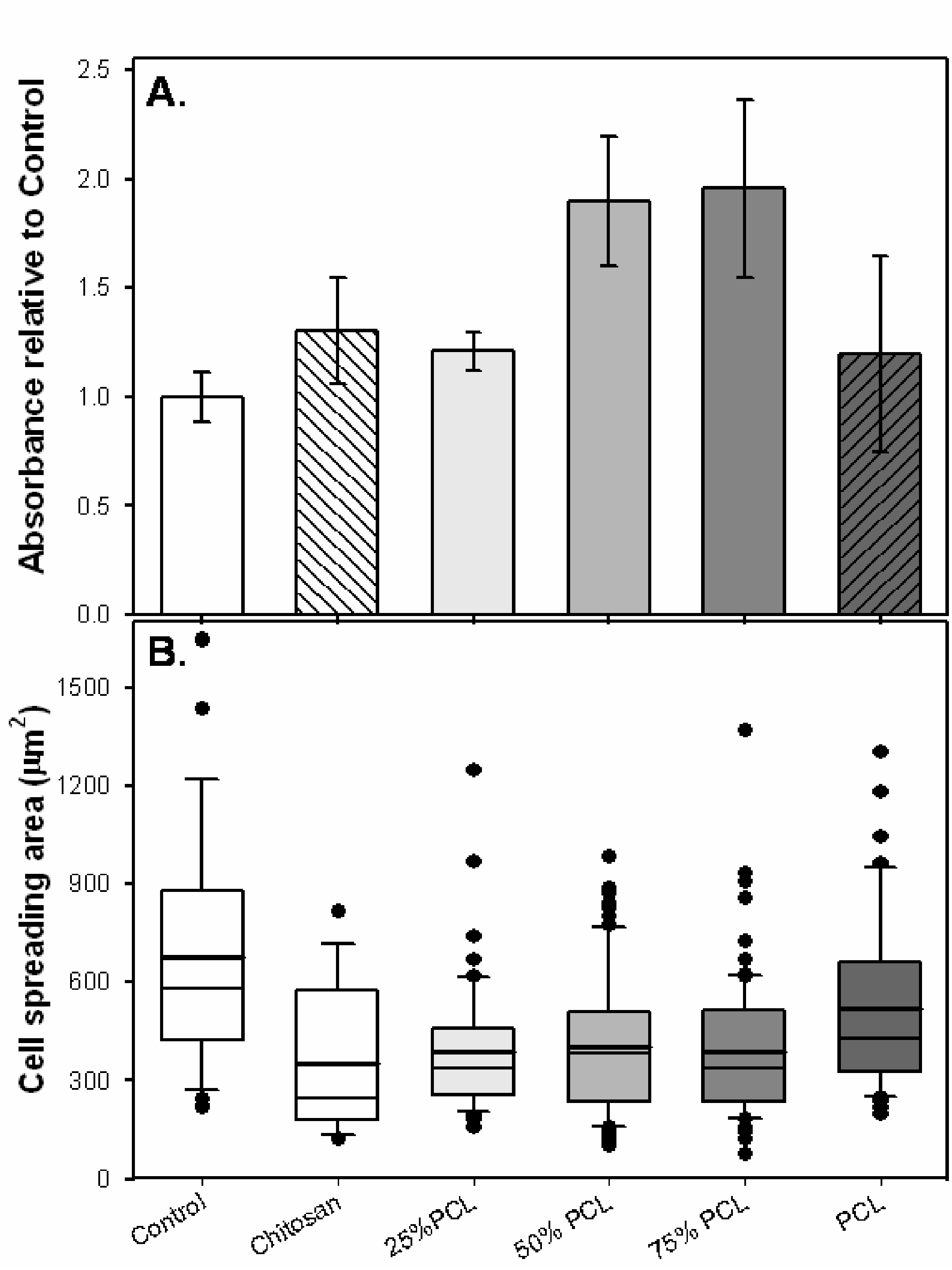

Figure 6.3. Influence of mixing chitosan and PCL on cellular activity of mouse embryonic fibroblasts. A) Viability of cells, normalized with respect to control. Data shown are standard deviations of three experiments. B) Box plots of cell spreading area.

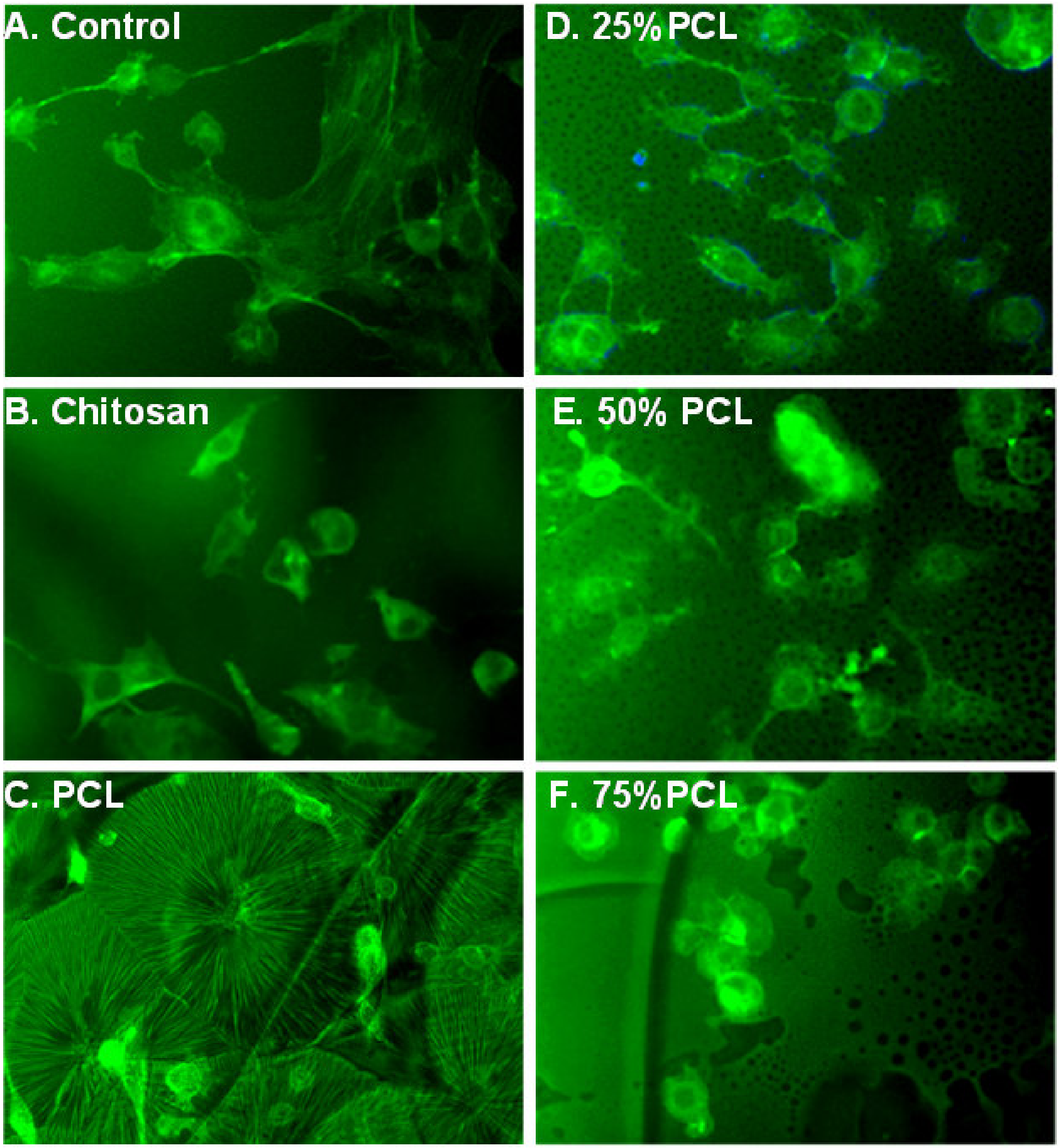

Figure 6.4. Influence of PCL mass ratio on cytoskeletal actin distribution of mouse embryonic fibroblasts on chitosan-PCL mixture membranes. Fluorescence micrographs after 48 hours.

chitosan scaffolds, sprouting blood vessels were seen around the scaffold **(Figure 6.5)**. Blood flow through these new vessels was also clearly visible. However, an interconnected vascular network was not witnessed and the chitosan scaffold started falling apart after 48 hours. New sprouting vessels anastomose (unite) with other existing vessels in 50% PCL scaffolds. This scaffold was also structurally more stable compared to chitosan or 25% PCL. 25% PCL scaffold showed results, similar to chitosan. In 75% PCL and pure PCL scaffolds, the biomaterial did not adhere to the CAM. The scaffolds could be seen merely floating on the top with no blood vessels going through them. The scaffolds themselves turned dark in color contrary to chitosan or other mixtures **(Figure 6.5)**.

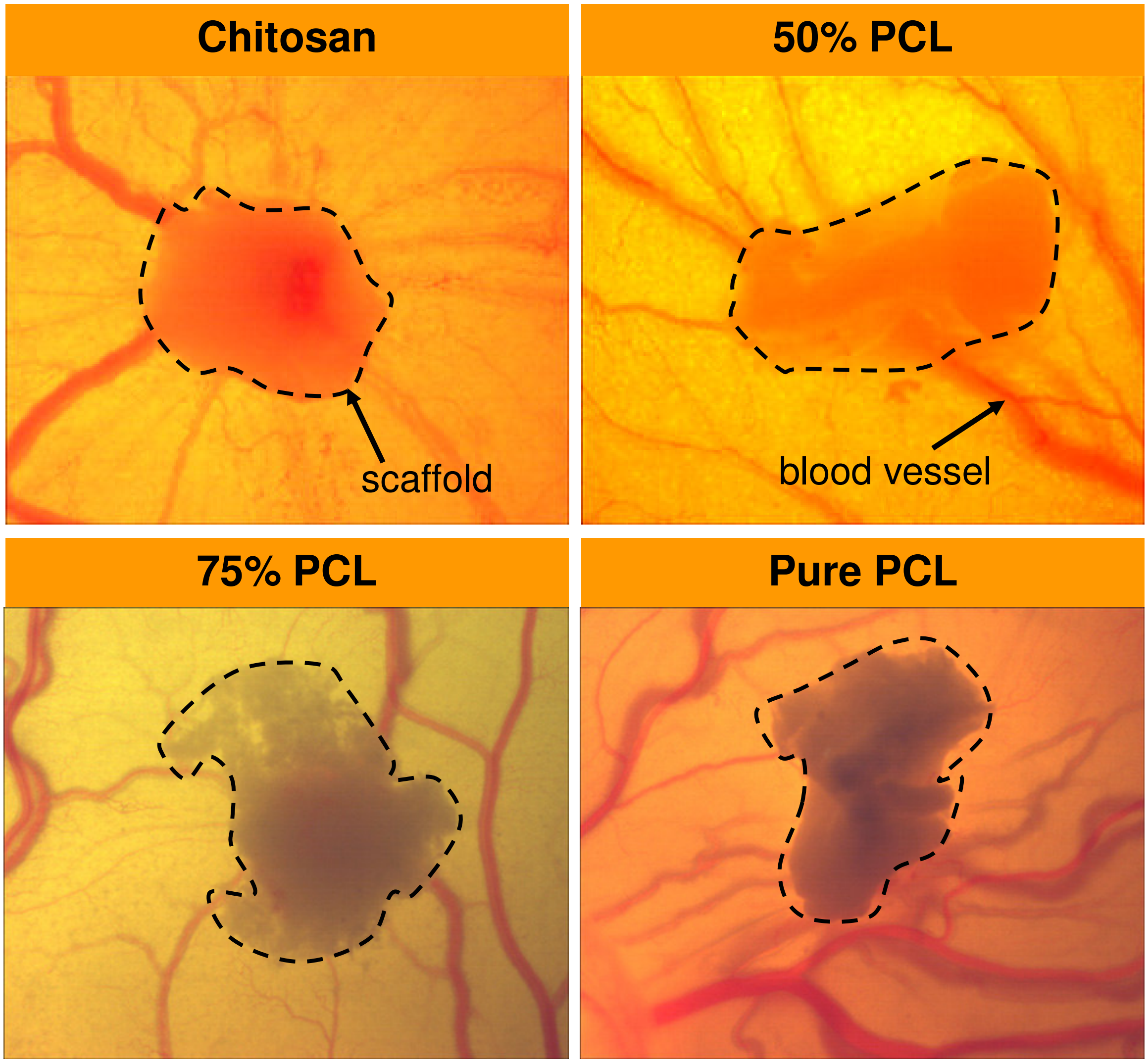

Figure 6.5. Influence of mixing chitosan and PCL on cytotoxicity and support to vasculature growth on chorioallantoic membranes. CAMs were wounded and chitoan-PCL mixture scaffolds were placed on top. Vasculature network on CAMs was imaged 30 hours after implantation.

6.4. DISCUSSION

Chitosan and PCL were brought together in a composite to combine the bioactivity of chitosan and the mechanical properties of PCL. The unique concoction of solvents used for dissolving these polymers was not cytotoxic. The composite showed improved cytocompatibility and support for cellular activity in 2D and 3D environments, compared to control or pure polymers. These results suggest that the cellular adhesion does not depend entirely on adhesive proteins and other receptors as both chitosan and PCL lack cell-binding domains.

Possible reasons for improved cellular activity could be alterations in a) chemical composition, b) charge density, c) water affinity, d) crystal structure and e) surface topography. For example, PCL membranes had a distinct spherulite type of morphology, compared to chitosan (**Figure 6.4**). Vasculature circuitry was completed only in 50% PCL mixtures suggesting that scaffold morphology might play an important role in angiogenesis. Further investigations are required to evaluate the effects of these factors.

6.5. CONCLUSION

In vitro cell culture results suggested that the unique acetic acid- water mixture used as solvents for common dissolution of chitosan and PCL did not cause cytotoxicity. In addition, 50% PCL and 75% PCL showed superior support to cellular viability and spreading, compared to individual polymers or control. CAM studies showed that in 3D form, these materials were not only cytocompatible, but also supported the formation of vasculature, suggesting that the 3D morphology was conducive to growth to tissues.

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CHAPTER VII

INFLUENCE OF MIXING CHITOSAN AND PCL ON ANTIBACTERIAL PROPERTIES

7.1. INTRODUCTION

Implantation sites in patients are often susceptible to infection due to contamination (Young and Sugarman 1988; Schierholz and Beuth 2001). Recent studies of regenerative surgical procedures using biocompatible cell embedding substances indicate that bacterial contamination of the tissue-engineered graft and the surrounding wound following surgery may adversely affect the formation of new tissue attachment (Gristina, Giridhar et al. 1993). Furthermore, introduction of any type of prosthesis or internal fixation tool may increase risk of infection due to introduction of a foreign material into the body (Gristina, Giridhar et al. 1993). Prevention or containment of bacterial contamination at the wound site may, therefore, significantly improve the clinical outcome of tissue engineering therapy. The development of a biomaterial that not only acts as an effective scaffold for tissue-engineered cell growth, but also demonstrates bacteriostatic qualities, is therefore crucial to improve graft survival and outcome in tissue engineering.

Chitosan is known for its bacteriostatic and bactericidal nature against numerous pathogenic organisms including *Porphyromonas. gingivalis* (Ikinci, Senel et al. 2002), *Escherichia coli* (Chung, Wang et al. 2003), *Actinobacillus actinomycetemcomitans* (Choi, Kim et al. 2001), *Pseudomonas aeroginosa, Staphylococcus aureus*

(Chung, Wang et al. 2003) and *Streptococcus mutans (Tarsi, Muzzarelli et al. 1997; Choi, Kim et al. 2001).* Due to the antibacterial activity, chitosan has been explored as an additive in mouth rinsing solution, edible films (Shahidi, Arachchi et al. 1999), wound dressing (Khor and Lim 2003), and coating medical devices to combat infection (Fujita, Kinoshita et al. 2004). In order to alter the biomechanical properties, chitosan was mixed with polycaprolactone (PCL) in this project. This was done by dissolving chitosan and PCL in different mass ratios in a unique acetic acid/water mixture (Chapter 3). *In vitro* biological studies showed that the formed mixtures and solvents employed were cytocompatible and increased the viability of eukaryotes (mouse embryonic fibroblasts) (Chapter 6). In this study, biological studies on chitosan/PCL mixtures were extended to prokaryotes.

Although, it is known that chitosan is antibacterial to several organisms, the antibacterial properties of chitosan in presence of other polymers have not been reported. PCL is susceptible to bacterial degradation (Engelberg and Kohn 1991; Jones, Djokic et al. 2005). Therefore, this study focused on assessing the anti-bacterial properties of chitosan/PCL mixtures against Gram-positive and Gram-negative bacteria. For this purpose, two representative oral pathogens, *Streptococcus mutans* and *Actinobacillus actinomycetemcomitans* were chosen. *S.mutans* is a Gram-positive spherical bacterium that is considered to be the most cariogenic (causing tooth decay) (Banas 2004), and *A.actinomycetemcomitans* is a rod-shaped coccobacillus involved in various forms of periodontitis and other non-oral infections such as septicemia and meningitis (Chaves, Jeffcoat et al. 2000).

Lastly, most of the studies that reported antibacterial properties of chitosan have tested it in solution form. It is suggested that in solution form, chitosan has a net positive charge, which binds the negatively charged bacterial cell walls and leaks the cytoplasm, causing death (Choi, Kim et al. 2001; Helander, Nurmiaho-Lassila et al. 2001). In this study, chitosan and chitosan/PCL mixtures were tested in membrane form, in order to investigate contact-dependent antibacterial activity. A novel assay was developed utilizing agar cultures to assess the contact-dependent adherence and proliferation of the adherent bacteria.

7.2. MATERIALS AND METHODS

Chitosan (85% DD, MW >310kD) and polycaprolactone (PCL) (MW 80kD) were purchased from Sigma Aldrich (St. Louis, MO). *Streptococcus mutans* (ATCC 25175, NCTC 10449) and *Actinobacillus actinomycetemcomitans* (ATCC 43719, NRRL 2501) bacteria were purchased from ATCC. All other chemicals used were of reagent grade.

7.2.1. Preparation of samples: Three milliliters of chitosan dissolved in 0.5M acetic acid was added to 10mL of PCL dissolved in glacial acetic acid, in a glass vial. The concentrations used were 2% chitosan with 0.2%, 0.6% and 1.8% PCL for 25% PCL, 50% PCL and 75% PCL mixtures, respectively. The mixtures were slightly warmed and stirred to form homogenous solutions. These solutions were cast in Teflon dishes for 24 hours in an oven at 55°C to form uniform membranes of approximately 100-120µm thickness. Membranes were cut into 2cm × 2cm strips, followed by immersion in 25mL of 90% ethanol each, for 15 minutes. The function of ethanol was to neutralize the acid as well as sterilize the membranes. Additionally, some chitosan membranes were neutralized with NaOH by immersion in 1N NaOH (4g/100mL) for 15 minutes, followed by sterilization in 90% ethanol for 15 minutes. Finally, the samples were washed thrice by immersion in sterile phosphate buffer saline (PBS) for 5 minutes each time.

7.2.2. Preparation of bacterial culture: Both the bacteria were grown for 48 hours in Brain Heart Infusion (BHI) broth at 37°C in aerobic conditions. Cultures in their early growth phase were used for anti-bacterial experiments.

7.2.3. Evaluation of bacterial proliferation in suspension cultures: Membrane strips neutralized and sterilized in 90% ethanol, were each suspended in 5mL of bacterial broths of known optical density (OD), taken in small glass vials. The vials were closed with rubber caps, and incubated at 37°C with constant gentle shaking. Transient changes in turbidity of the broths were measured in triplicate samples at 3,6,12 and 24 hours by withdrawing 0.5mL of broth, diluting to 1mL with deionized water and measuring the OD using a spectrophotometer at 660nm wavelength. Suspension cultures without any membranes were used as control. Additionally, the influence of neutralization solvent on bactericidal activity was assessed by testing NaOH- neutralized chitosan membranes in the same way.

7.2.4. Evaluation of bacterial adhesion in suspension cultures: Membrane strips from suspension cultures were retrieved after 24 hours and fixed in 3.7% paraformaldehyde for 30 minutes followed by washing with PBS. The wet membranes were air-dried in a vacuum desiccator for 24 hours prior to sputter coating with gold for 1 minute. Bacterial adherence on the membranes was observed by Jeol 6360 Scanning Electron Microscope (SEM) (Jeol USA Inc., Peabody, MA), and images were obtained at representative locations.

7.2.5. Evaluation of contact-dependent bacterial adhesion and proliferation on agar cultures: Dense 'mats' of bacteria were generated on sterile BHI agar plates by spreading 25μL of turbid bacterial cultures (optical density 1.5 or more) over approximately 2cm × 2cm area and incubating at 37°C for 12 hours. Membrane strips

(2cm × 2cm size) neutralized and sterilized in 90% ethanol, were placed on these mats and gently pressed to ensure complete contact of the membranes with the mats. Visible changes in the morphology of the mats underneath the membranes were monitored with respect to control (mats with no membranes on top) and digital images were obtained.

After 24 hours, one set of membranes from each group were removed and analyzed by SEM. Another set was rehydrated by adding few drops of media, then gently removed and placed on a clean agar plate, bottom side facing down, so that the surface of membranes that had been in contact with the mats was in contact with the fresh agar. Bacterial growth accompanying the membranes was monitored as before. After 24 hours, membranes were removed and analyzed by SEM. In addition to mat cultures, membranes suspended in bacterial broth for 24 hours were also seeded on an agar plate (without any mats) to check for viability of adherent bacteria, if any.

7.2.6. Statistical analysis: All experiments were repeated three or more times with triplicate samples in each experiment. Significant differences between experimental groups were evaluated using a one-way Analysis of Variance (ANOVA) with 99% confidence interval.

7.3. RESULTS

7.3.1. Influence of mixing on bacterial proliferation in suspension cultures: The bactericidal properties of chitosan against Gram- positive *S.mutans* were tested by suspending the membrane in bacterial broth and incubating at appropriate conditions. Transient changes in optical density of the broth indicated that except 50% PCL, all other biomaterials showed a decreased growth of bacteria in suspension, with respect to control (**Figure 7.1**). There were no significant differences in the optical densities of 25% PCL and 75% PCL suspensions. At any given time, suspensions containing chitosan membranes showed the least growth of bacteria. Nevertheless, the growth of bacteria in presence of these membranes indicated that chitosan, PCL or their mixtures are not bactericidal. When the experiments were repeated with Gram- negative *A.actinomycetemcomitans*, growth was observed in all suspensions including chitosan, indicating that the membranes were not bactericidal to this pathogen either (**Figure 7.1**). Nevertheless, in comparison to other groups, chitosan showed lower growth rates of *A.actinomycetemcomitans,* similar to *S.mutans*. Interestingly, unlike *S.mutans*, the growth rate of *A.actinomycetemcomitans* in presence of these membranes was higher than in control. This indicated that the anti-bacterial activity of chitosan was more effective against *S.mutans* than against *A.actinomycetemcomitans* in suspensions.

7.3.2. Influence of mixing on bacterial adhesion in suspension cultures: When the adhesion of *S.mutans* to the membranes was observed under SEM, results indicated that there was negligible adhesion on chitosan membranes (**Figure 7.2**). Presence of PCL significantly increased bacterial adhesion on the membranes. Similar results were observed with *A.actinomycetemcomitans* (**Figure 7.2**). On the 75% PCL membrane, chains of rod-shaped *A.actinomycetemcomitans* appeared fragmented, due to cracking of underlying substrate during sample preparation for SEM, indicating that probably bacteria adhered strongly to the membranes.

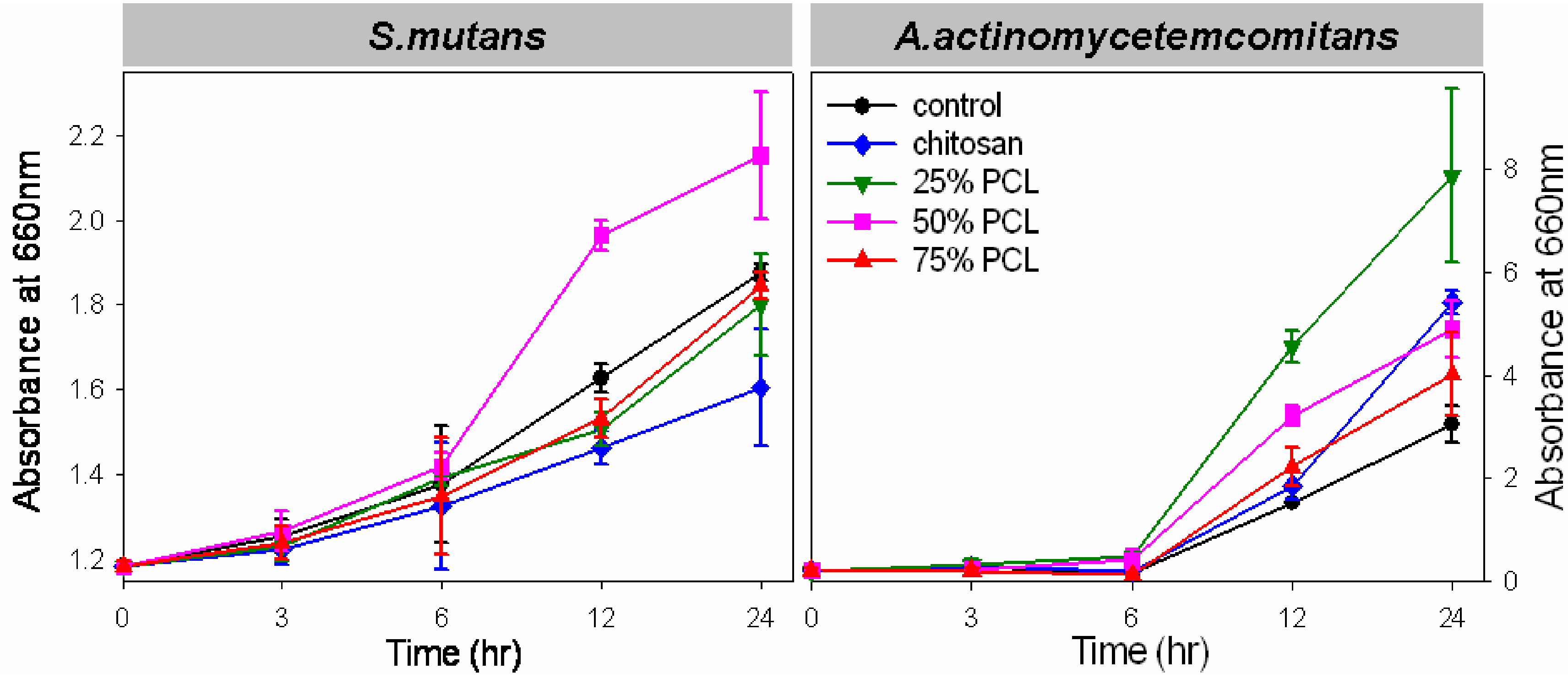

Figure 7.1. Influence of mixing with PCL on antibacterial property of chitosan membranes in suspension cultures. Membranes were suspended in bacterial broths, and incubated aerobically at 37°C for 24 hours. Transient changes in optical density of broth were measured at 660nm wavelength.

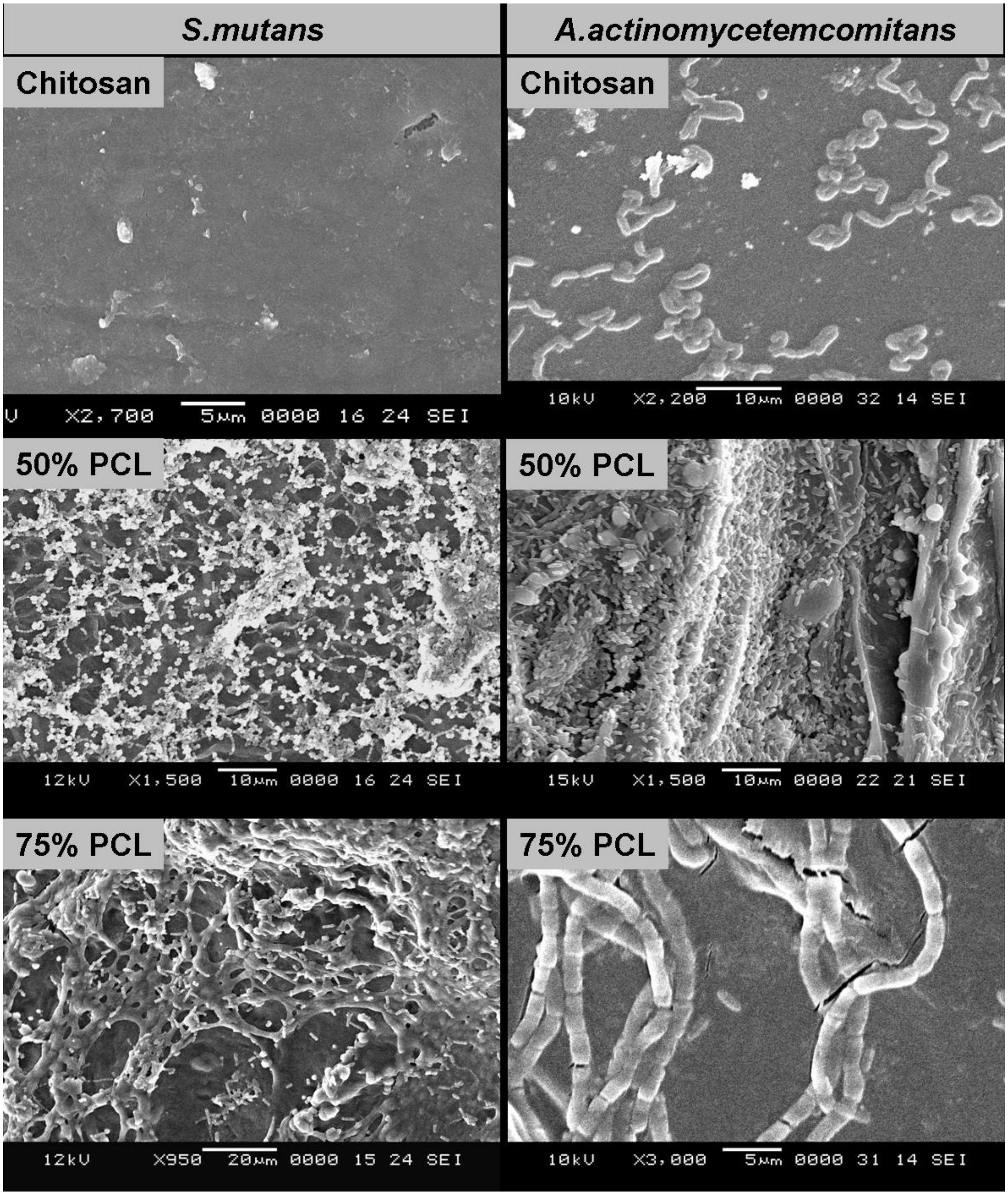

Figure 7.2. Influence of mixing with PCL on antibacterial adhesion of chitosan membranes in suspension cultures. Membranes were suspended in bacterial broths and incubated aerobically at 37C for 24 hours. Membranes were withdrawn and analyzed for bacterial adhesion by SEM.

7.3.3. Influence of neutralization solvent on antibacterial properties of chitosan: The OD of *A.actinomycetemcomitans* cultures containing NaOH-neutralized chitosan membranes ($(A/A^{\circ})_{chitosan}$) did not show significant difference relative to control cultures ($(A/A^{\circ})_{control}$) at different time points (**Figure 7.3**). However, OD of cultures containing ethanol-neutralized chitosan membranes showed significant increase relative to control. Interestingly, ethanol-neutralized membranes showed negligible adhesion of *A.actinomycetemcomitans* whereas NaOH-neutralized membranes showed significant bacterial adhesion (**Figure 7.3)**. This trend in proliferation and adhesion of bacteria suggests that the antibacterial properties of chitosan are dependent on surface characteristics and chemistry.

7.3.4. Influence of mixing on contact-dependent bacterial adhesion and proliferation on agar cultures: To further understand the influence of surface dependent antibacterial properties of chitosan, chitosan/PCL mixture membranes were placed on bacterial mats grown on agar plates and analyzed for anti-bacterial activity. After 24 hours, there was minimal growth of *A. actinomycetemcomitans* under chitosan membranes, and the transparency of the membranes was also retained, suggesting that there was no proliferation on the chitosan surface (**Figure 7.4C**). The bacterial colonies spread out to the surroundings of PCL and chitosan/PCL mixture membranes and appeared cloudier, although growth underneath could not be observed due to the opaque nature of the membranes. SEM analysis of these membranes confirmed that there was negligible adhesion to chitosan surfaces when compared to the PCL and chitosan/PCL mixture membranes (**Figure 7.5**). To further understand the viability and proliferative ability of

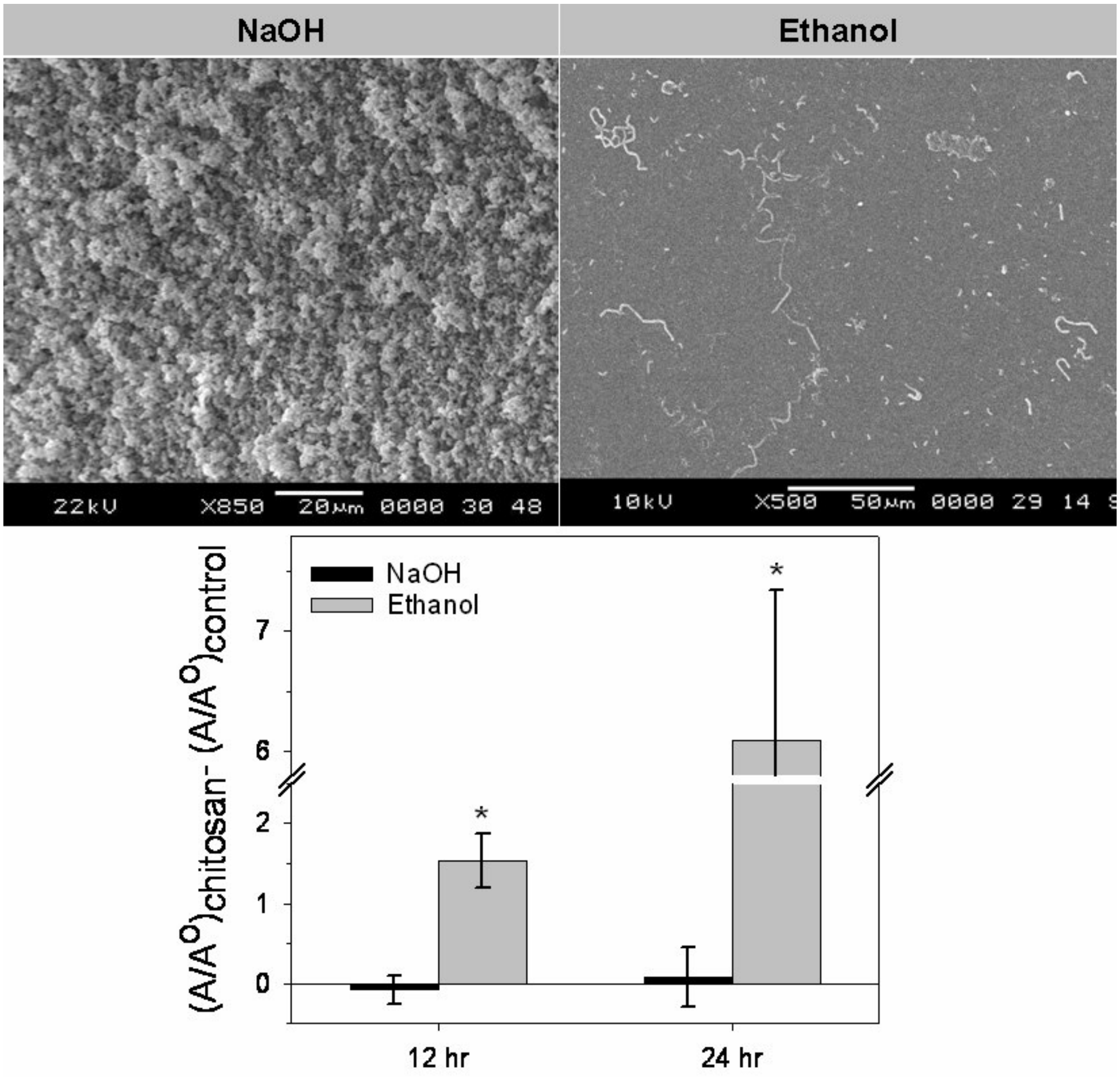

Figure 7.3. Influence of neutralization solvent on antibacterial properties of chitosan membranes to *A. actinomycetemcomitans* in suspension cultures. Membranes were neutralized with 1N NaOH, sterilized with 90% ethanol, and incubated aerobically in bacterial broths at 37°C. Absorbance was normalized with respect to 0hr. *P<0.01 relative to NaOH-neutralized samples.

adherent bacteria, these membranes were transferred to a fresh agar plate with no mats. There was no visible bacterial growth underneath the chitosan membranes, confirming contact-dependent anti-bacterial property of chitosan surface (**Figure 7.4D**).

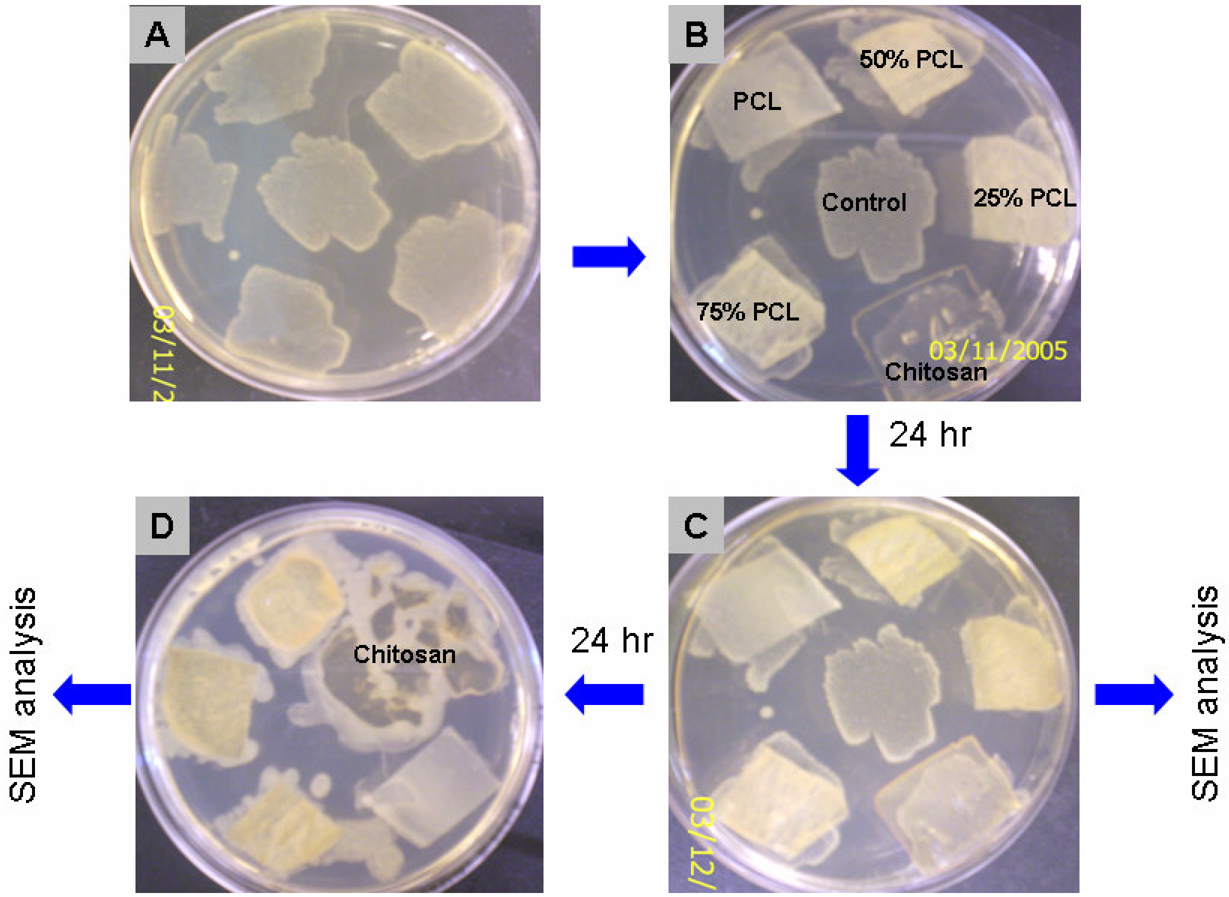

Figure 7.4. Novel agar culture assay to study contact dependent growth of *A. actinomycetemcomitans* on chitosan- PCL mixture membranes. A) Dense mats without membranes. B) Ethanol sterilized membranes seeded on top of dense mats. C) Morphology of mats after 24 hours. D) Membranes peeled and seeded on fresh agar plate.

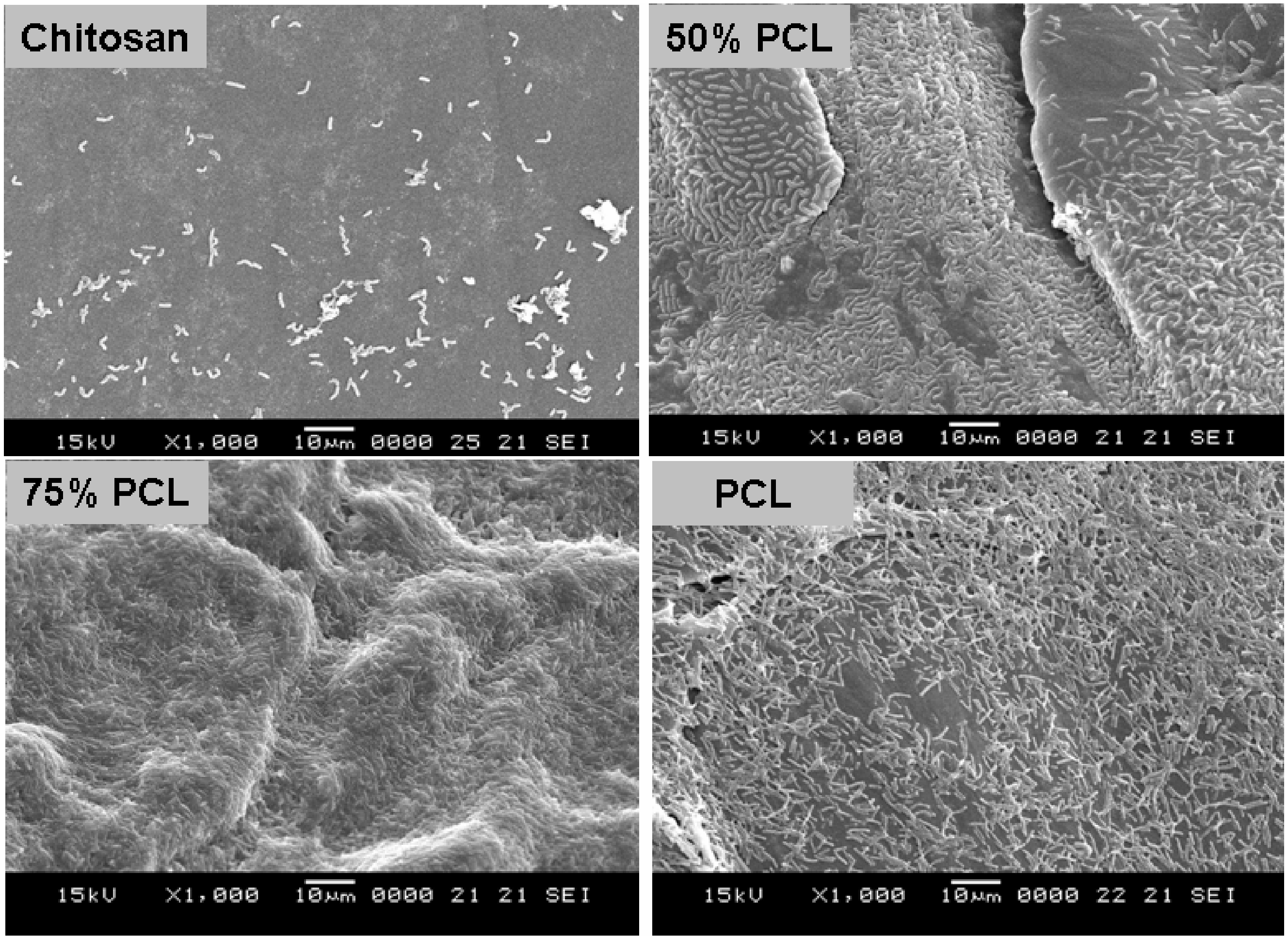

Figure 7.5. SEM images showing adhesion of *A.actinomycetemcomitans* on membranes from Figure 7.4C.

There was bacterial growth surrounding PCL and chitosan/PCL mixture membranes, suggesting that attached bacteria were proliferative. In addition, SEM analysis (**Figure 7.6**) verified the presence of bacteria on these membranes, similar to **Figure 7.5**. Similar experiments were performed with *S.mutans* and results were identical to those obtained with *A. actinomycetemcomitans*.

To confirm the observations in suspension culture, membranes incubated in the bacterial broth for 24 hours were also tested on agar without any mats (**Figure 7.7A**). This ensured that only those bacteria that adhered naturally to the membranes in suspension were tested for contact-dependent proliferation, instead of forced adhesion as in **Figure 7.4B**. After 24 hours, there was no growth accompanying chitosan membranes, unlike the mixtures and PCL membranes (**Figure 7.7B**). These results confirmed that the anti-bacterial nature of chitosan is contact-dependent.

7.4. DISCUSSION

There are two aspects to anti-bacterial nature, bacteriostatic and bactericidal. Bactericidal refers to the ability of the material to kill microorganisms, whereas bacteriostatic implies the ability to arrest their growth. Chitosan is a positively charged hydrophilic polysaccharide, known for its antibacterial properties against several organisms. PCL is uncharged hydrophobic polyester, without any documented evidence of antibacterial nature (Wiseman, Domb et al. 1998). In this study, chitosan and PCL were combined in varying mass ratios to form composite membranes. The resistance to bacterial proliferation (bacteriostatic or bactericidal) as well as bacterial adhesion on the surface or surrounding media was measured.

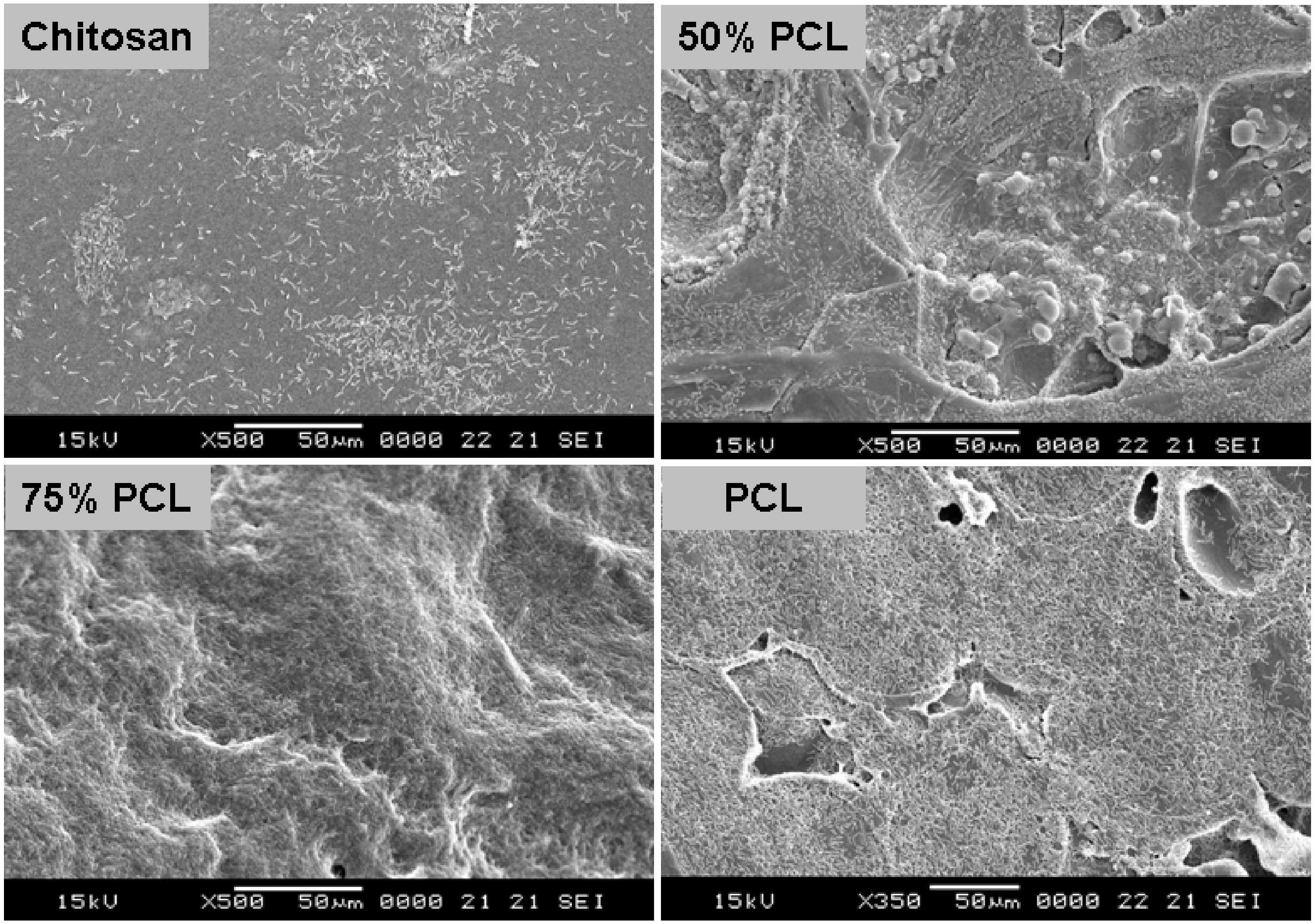

Figure 7.6. SEM images showing adhesion of *A.actinomycetemcomitans* on membranes from Figure 7.4D.

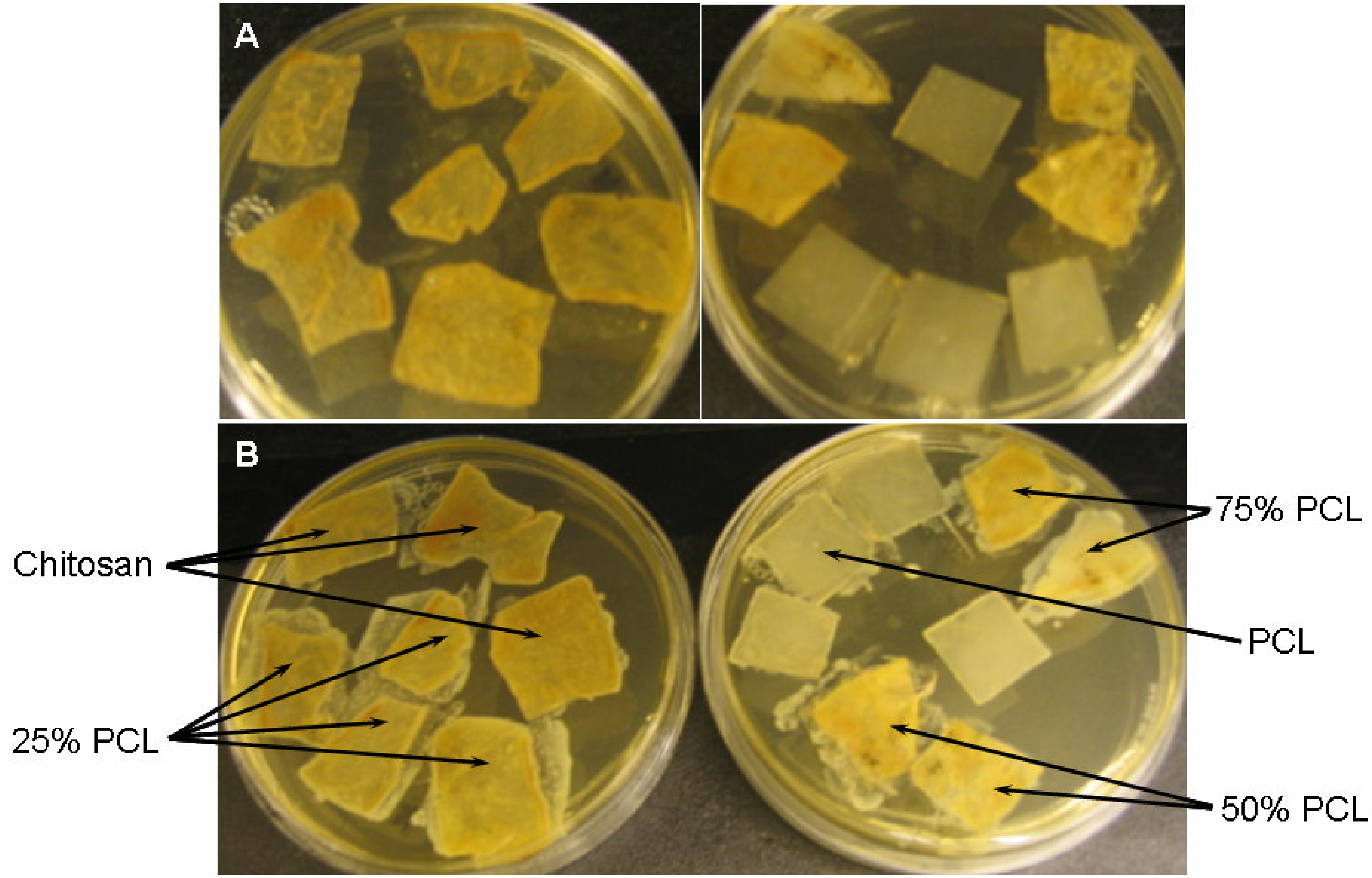

Figure 7.7. Contact-dependent growth of *A. actinomycetemcomitans*. Membranes were first incubated in *A. actinomycetemcomitans* broth, and then seeded on agar plates. Digital images obtained after A) 0 hours, and B) 24 hours.

Results suggest that the antibacterial nature of chitosan to both Gram-positive and Gram-negative bacteria was compromised by mixing with PCL. The proliferation of *S.mutans* and *A.actinomycetemcomitans* in suspensions increased with the addition of PCL. Adhesion of these bacteria which was negligible on chitosan also increased drastically on chitosan/PCL membranes. There was no clear correlation between the mass ratios of chitosan and PCL, and the antibacterial activity. The reasons for loss of antibacterial properties could be reduction in surface cationic charge of chitosan, which is believed to cause bacterial cell death, or due to changes in hydrophilicity. Charge and hydrophilicity of chitosan are related, as protonation of the amine group in water produces cationic ammonium ion (Hu, Jiang et al. 2002). Recent studies have attempted at correlating the antibacterial activity to water solubility of chitosan (Qin, Li et al. 2006). It was found that water-insoluble chitosan, dissolved in acidic medium, exhibited the best antimicrobial action.

Interestingly, the bactericidal potency of chitosan was also dependent on the strain(No, Lee et al. 2003). Chitosan was more potent against gram-positive *S. mutans* than against gram-negative *A.actinomycetemcomitans*, similar to literature reports (No, Park et al. 2002). This could probably be attributed to the presence of lipopolysaccharide layer that protects the structural integrity of Gram-negative bacteria. The lipopolysaccharide also increases the overall negative charge of the bacterial cell wall which might have caused the strong adhesion of *A.actinomycetemcomitans* to the cationic chitosan surface.

Chitosan is soluble in water under acidic conditions (pH<6.3). Therefore, chitosan based matrices are usually neutralized to remove acidity, prior to hydration.

Neutralization can be done either by using NaOH or ethanol. Although these two solvents are bases with extremely high affinity for water, they have different physical and chemical properties, which could alter the surface properties of chitosan in different ways, and to different degrees. As expected, neutralization by ethanol allowed negligible bacterial adhesion on chitosan membrane, whereas NaOH neutralized membranes showed the opposite trend.

Results of this work also suggest that the antibacterial properties of chitosan may be contact dependent as it allows proliferation of bacteria away from it in suspension while inhibiting bacterial adhesion on its surface, similar to literature reports (Fujita, Kinoshita et al. 2004). A novel assay was adapted to directly assess the contact-dependent antibacterial activity of chitosan. By placing the membranes directly on colonies of bacteria (mats), it was ensured that the bacteria could proliferate only on the surface of chitosan and chitosan/PCL mixture membranes. Proliferation of the adherent bacteria by serial plating indicated that there was minimal bacterial adhesion and viability on chitosan, whereas the mixtures and pure PCL membranes allowed bacterial proliferation and viability to a large extent. This can be attributed to the altered surface characteristics of chitosan as well as the bacterial susceptibility of PCL (Jones, Djokic et al. 2005).

In this study, apart from surface charges and hydrophilicity, there could be other reasons for loss of antibacterial properties of chitosan/PCL mixture membranes. Important among those are surface topographical aspects such as roughness. A number of studies with other macromolecules have shown that chemical and mechanical properties of matrices such as edges, grooves, hydrophilicity, presence of adhesion domains, roughness/ nanotopographies, and stiffness, influence eukaryotic cellular processes in 2D matrices (Rajnicek, Britland et al. 1997; Curtis and Wilkinson 1999; Ranucci, Kumar et al. 2000; Balgude, Yu et al. 2001).

7.5. CONCLUSION

In summary, this study shows that chitosan-mediated antibacterial activity is contact-dependent and that growth of bacteria occurs away from the membrane in suspension. Mixing chitosan with other non-antibacterial polymers such as PCL may compromise its antibacterial activity. Lastly, the choice of neutralization media of chitosan greatly affects its anti-bacterial activity. Neutralizing chitosan with ethanol, rather than with NaOH, maybe a better option for tissue engineering applications, where it is necessary to abate bacterial colonization on the matrix while promoting tissue cell growth.

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CHAPTER VIII

MOLECULAR INTERACTIONS BETWEEN CHITOSAN AND PCL, AND CHANGES IN PHYSICOCHEMICAL PROPERTIES

8.1. INTRODUCTION

In this project, two biomaterials, chitosan and PCL were combined to obtain a composite that has the biological properties of chitosan with the mechanical strength of PCL. This was achieved by dissolving both the polymers in varying mass ratios, in a unique acetic acid-water mixture. Solution mixing was the chosen method to combine chitosan and PCL in this project, as the retention of their unique individual properties could be ensured. These composite membranes showed improved biomechanical properties, in comparison to the pure polymers, as discussed in previous chapters.

The focus of this study was to investigate the factors responsible for alterations in the biomechanical properties of chitosan/PCL mixtures. As there were no noticeable chemical modifications taking place by solvent dissolution, it was suspected that alterations in physicochemical properties might have influenced the biomechanical performance of these composites. Additionally, chitosan and PCL mixtures present a model system for studying composites of two semi-crystalline polymers that differ in charge distribution, as chitosan is cationic in nature and PCL is uncharged. Therefore, the molecular interactions were investigated by fourier transform infrared spectroscopy (FTIR) and changes in physicochemical properties such as crystallinity and crystal structure were studied by differential scanning calorimetry (DSC), dynamic mechanical and thermal analysis (DMTA), and wide angle x-ray diffraction (WAXD). Additionally, since the biological properties of chitosan and scaffolds in general are surface dependent, changes in surface topography and charge density of membranes were investigated by atomic force microscopy (AFM) and electron force microscopy (EFM), respectively.

8.2. MATERIALS AND METHODS

Chitosan (~85% DD, MW >310kD) and polycaprolactone (PCL) (MW 80kD) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals used were of reagent grade.

8.2.1. Preparation of samples: Three milliliters of chitosan (MW>310kD) dissolved in 0.5M acetic acid was mixed with 10mL of PCL (MW 80kD) dissolved in glacial acetic acid in a glass vial. The concentrations used were 2% chitosan with 0.2%, 0.6% and 1.8% of PCL for 25% PCL, 50% PCL and 75% PCL mixtures, respectively. The mixtures were warmed slightly and stirred to form homogenous solutions. These solutions were cast in Teflon dishes for 24 hours in an oven at 55°C to form uniform membranes of approximately 100-120μm thickness.

8.2.2. Evaluation of molecular interactions by FTIR: Membranes were probed using a Thermo Nicolet IR300 FT-IR Spectrometer (Thermo Electron Corporation, Madison, WI). Samples were mounted on a film holder and spectral scans of films were obtained at ambient conditions at a resolution of 4cm^{-1} with an accumulation of 8 scans, using the associated EZ-OMNIC software. Data was plotted using SigmaPlot 9 software (SPSS Science, Chicago, IL) and peaks were analyzed for functional groups.

8.2.3. Evaluation of crystal structure by WAXD: Changes in crystal structure of chitosan and PCL in the mixtures were analyzed using a Rigaku-MSC Wide-Angle X-Ray Diffractometer (Rigaku/MSC, The Woodlands, TX) in D-MAX-A mode with an MDI databox. X-rays were generated using a 12kV rotating anode generator and the goniometer was rotated from 2° to 40° at a speed of 2°/min.

8.2.4. Evaluation of miscibility and interactions by DSC: Thermal properties of various scaffolds were characterized using a Perkin-Elmer DSC 7 (Perkin Elmer, Boston, MA). Nitrogen at the rate of 20mL/min was used as purge gas. Membranes in the amount of 5-10mg were sealed in aluminum pans and heated up to 100°C at the rate of 10°C/min. Melting temperature of PCL (T_m) was taken as the temperature at which the endothermic peak occurred. Pure chitosan samples were heated upto 325°C.

8.2.5. Dynamic Mechanical and Thermal Analysis: Thermo-mechanical analysis was performed on dry membranes of 20mm×6mm size using a Seiko DMS 200 DMTA. Samples were heated from -100°C to 160°C at a rate of 2°C/min and measurements were made at 0.1, 1.0 and 10Hz frequencies simultaneously in tension mode under nitrogen atmosphere. Changes in storage modulus (E') and loss factor (tanδ) were recorded. The peak heights of tanδ curve were calculated using SigmaPlot 9 software.

8.2.6. Evaluation of surface roughness and morphology by AFM: Surface analysis of membranes was done by AFM using a DI Nanoscope IIIa Multimode Scanning Probe Microscope (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA) at ambient conditions. Sample films were attached onto iron AFM substrate disks using double-sided tape. Topographic images were obtained in tapping mode using commercial silicon microcantilever probes (MikroMasch, Portland, OR) with a tip radius of 5-10nm and spring constant 2-5N/m. The probe oscillation resonance frequency was ~120 KHz and scan rate was 1Hz. Images were captured at different locations and the roughness factors were calculated using associated software (Nanoscope, version 5). The roughness factor R_q is the root mean square average of 'n' number of height deviations (Z_i) taken from the mean data plane:

$$Rq = \sqrt{Z_i^2 / n} \ .$$

8.2.7. Evaluation of surface charge distribution by EFM: Digital Instruments Multimode Scanning Probe Microscope equipped with Nanoscope IIIa controller (Veeco, Santa Barbara, CA) was used to record the electrical force microscopic images. Samples are prepared by depositing 1-2 drops of polymer solutions on a clean and dry 15mm x 15mm silicon wafer and air-drying. A Ti-Pt coated silicon AFM cantilever with an integrated tip (MikroMasch, Oregon) vibrates near its resonant frequency of 75 kHz and scans over the sample surface. After acquiring topographical height using tapping mode, the tip makes a second pass over each line, at a height of 60nm relative to the sample. During each second pass, the tip is biased at a voltage of -4V and the frequency shift (Δf) between the mechanical drive and the cantilever's motion is recorded.

8.2.8. Statistical analysis: All experiments were repeated three or more times, in triplicate samples. Significant differences between experimental groups were evaluated using a one-way Analysis of Variance (ANOVA) with 99% confidence interval.

8.3. RESULTS

8.3.1. Molecular Interactions between chitosan and PCL: FTIR spectra are presented in three frequency ranges in **Figure 8.1A** showing the typical absorption peaks of the functional groups present in chitosan and PCL (Pawlak and Mucha 2003; Elzein, Nasser-Eddine et al. 2004). These peaks were also observed in all the mixture spectra although with varying intensities based on their composition. Characteristic peaks of chitosan were more distinguishable in 25% PCL whereas those of PCL were better expressed in 50% and 75% PCL mixtures. The amine group of chitosan could potentially form an amide bond with the carbonyl group of ester groups in the chain or carboxylic acid groups at the end of chains of PCL (**Figure 8.1B**). In such a case, the absorption peak of carbonyl stretch would shift from 1725 cm^{-1} to a lower value (approximately 1630-1680cm^{-1}) and the two peaks corresponding to amine group of chitosan would convert to one weak band as the primary amine changes to secondary form. These changes were not observed in the mixture spectra ruling out the possibility of the amide bond formation. The C-N bond stretch in the new amide bond, if any, could not be distinguished from the existing C-N bonds in chitosan at 1000-1350cm^{-1} (Pavia, Lampman et al. 2000). These results implied that there was no detectable chemical bonding between chitosan and PCL in the formed mixtures, but they only coexisted.

8.3.2. Influence of mixing on crystal structure: WAXD spectrum of chitosan showed characteristic peaks (**Figure 8.2A**) at 10° and 20.5° (Min, Lee et al. 2004). Both the peaks appeared only in 25% PCL mixture and were broad and weak. Diffraction spectrum of PCL (**Figure 8.2B**) showed sharp and well-defined characteristic peaks at 21.5°, 22° and 23.5° (Piacibello, Sanavio et al. 1997). These peaks were present in 75% PCL spectrum at the same angles with slightly reduced intensity. Fifty percent PCL mixture (**Figure 8.2A**) showed all peaks of PCL as well as chitosan peak at 10°,

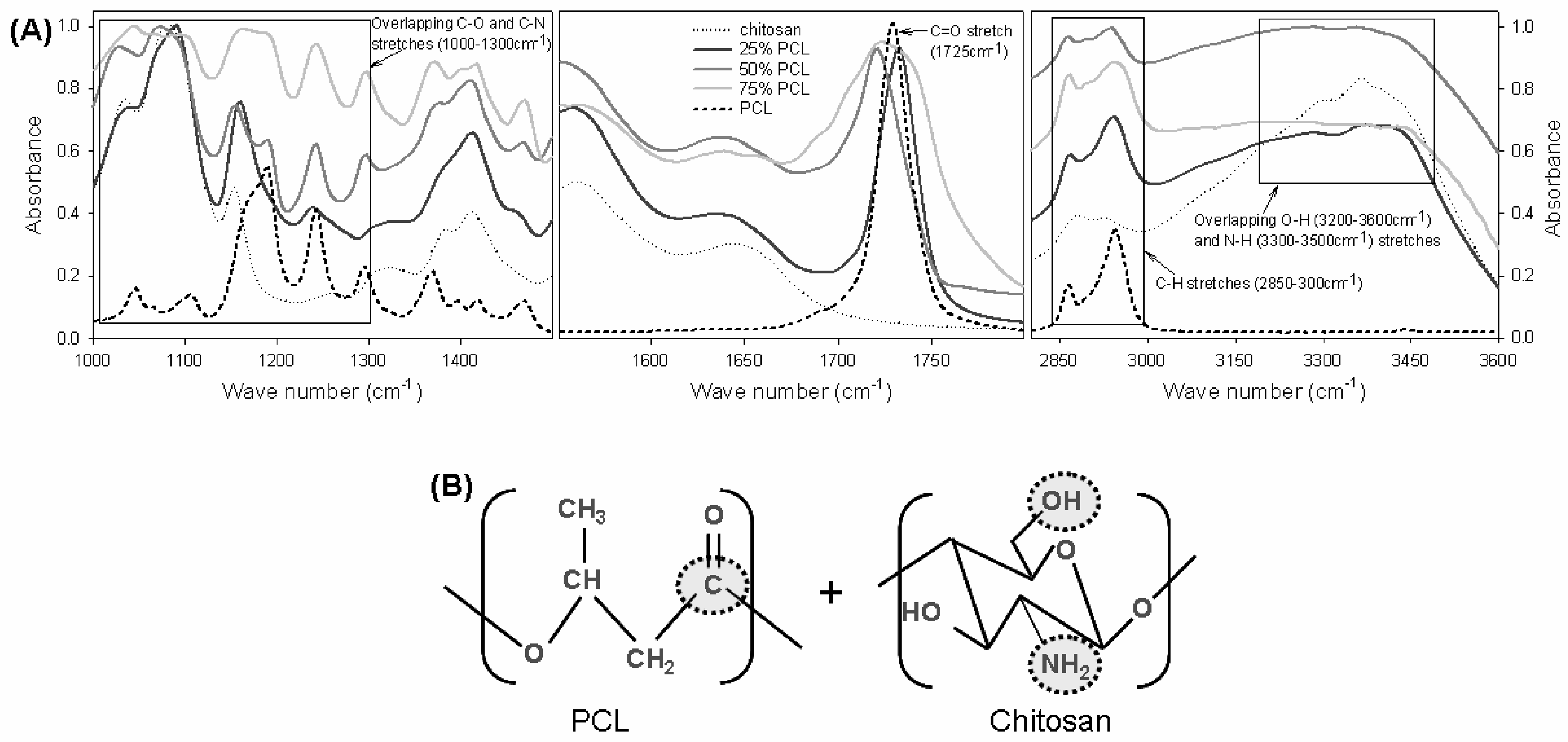

Figure 8.1. A) FTIR spectra of chitosan/PCL mixture membranes in three frequency regions. B) Functional groups of chitosan and PCL with possibility of interaction.

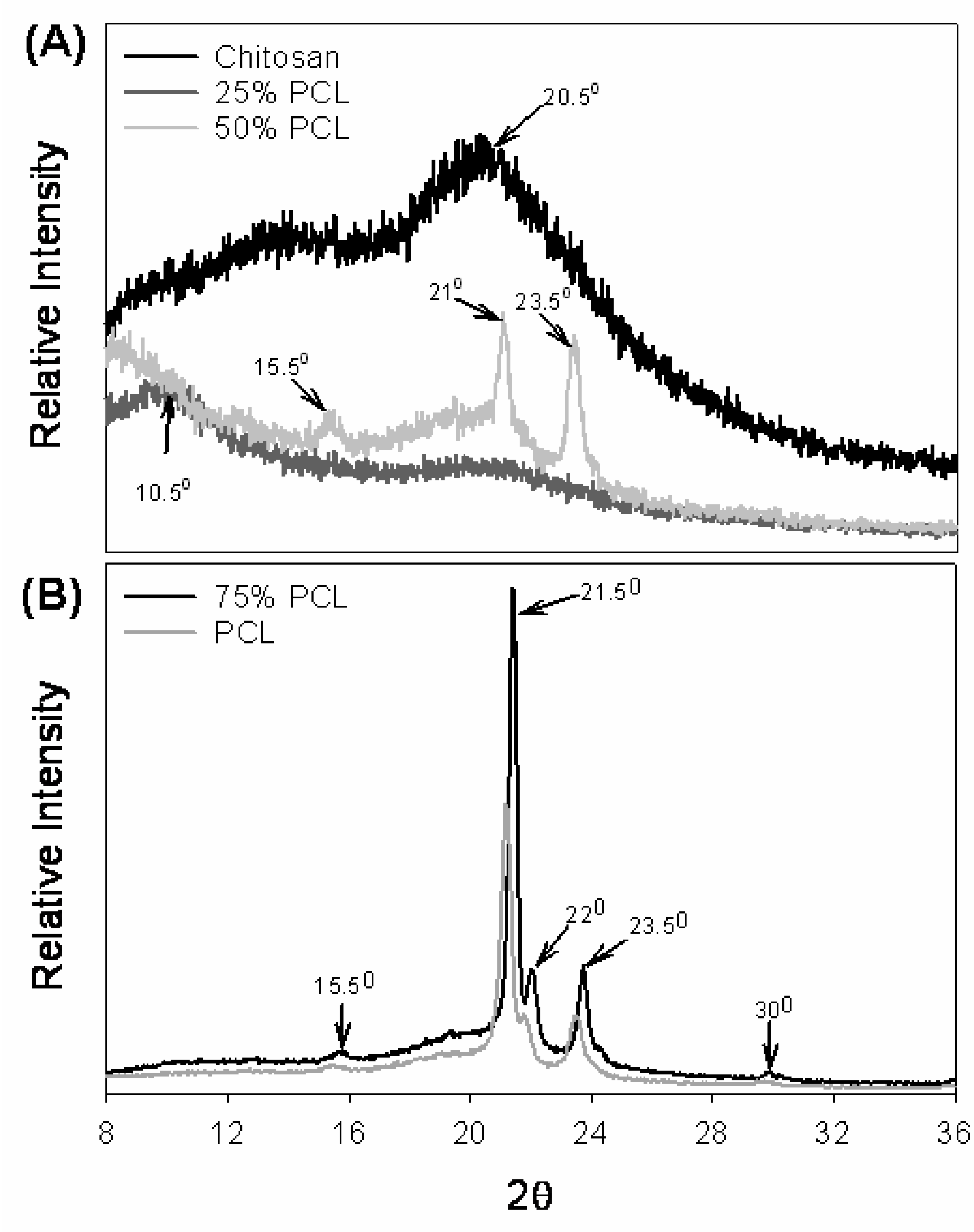

Figure 8.2. Influence of mixing chitosan and PCL on their crystal structure. WAXD spectra of chitosan, PCL and mixture membranes.

although with much reduced intensity. Absence of any additional peaks or shift in the diffraction angles indicated that the crystal structure of chitosan and PCL is unchanged in the mixtures. The crystal structure of mixtures was influenced by the dominant component i.e. 25% PCL was similar to chitosan, whereas 50% and 75% PCL had structure similar to PCL.

8.3.3. Miscibility and phase transitions in mixtures: There are wide variations in the reported values of glass transition temperature (T_g) of chitosan in literature (Suyatma, Tighzert et al. 2005). Most papers do not report T_g of chitosan while others reported at temperatures ranging from 140°C to 200°C. In this study, DSC thermogram of chitosan membranes obtained from first scan (**Figure 8.3)** showed a typical exothermic peak at

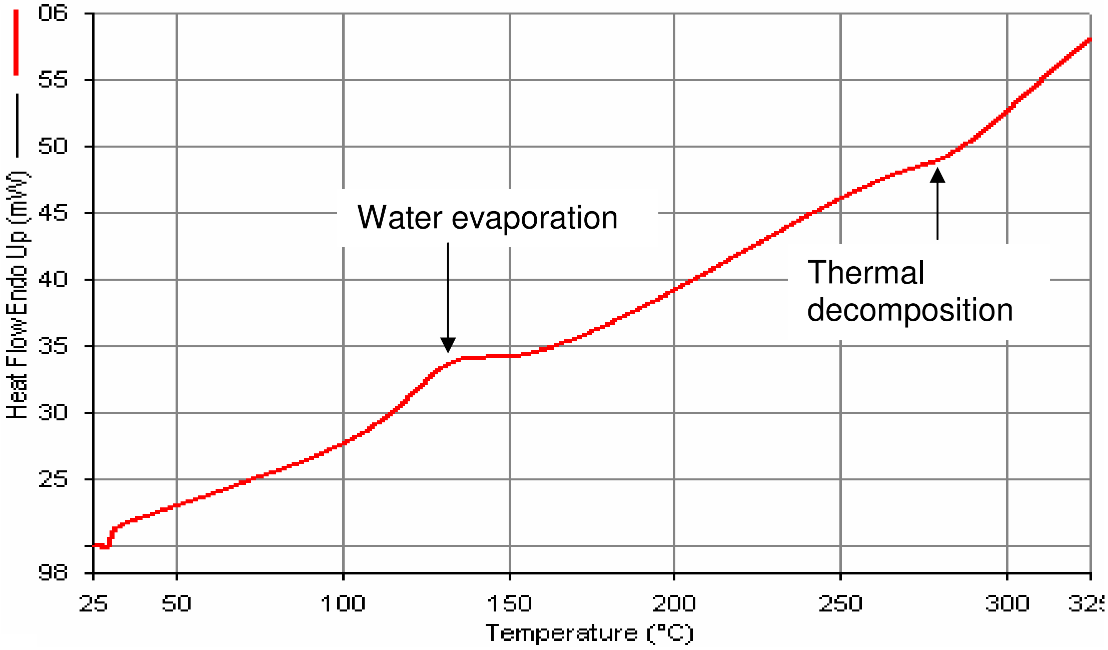

Figure 8.3. DSC thermogram of chitosan membrane from first scan.

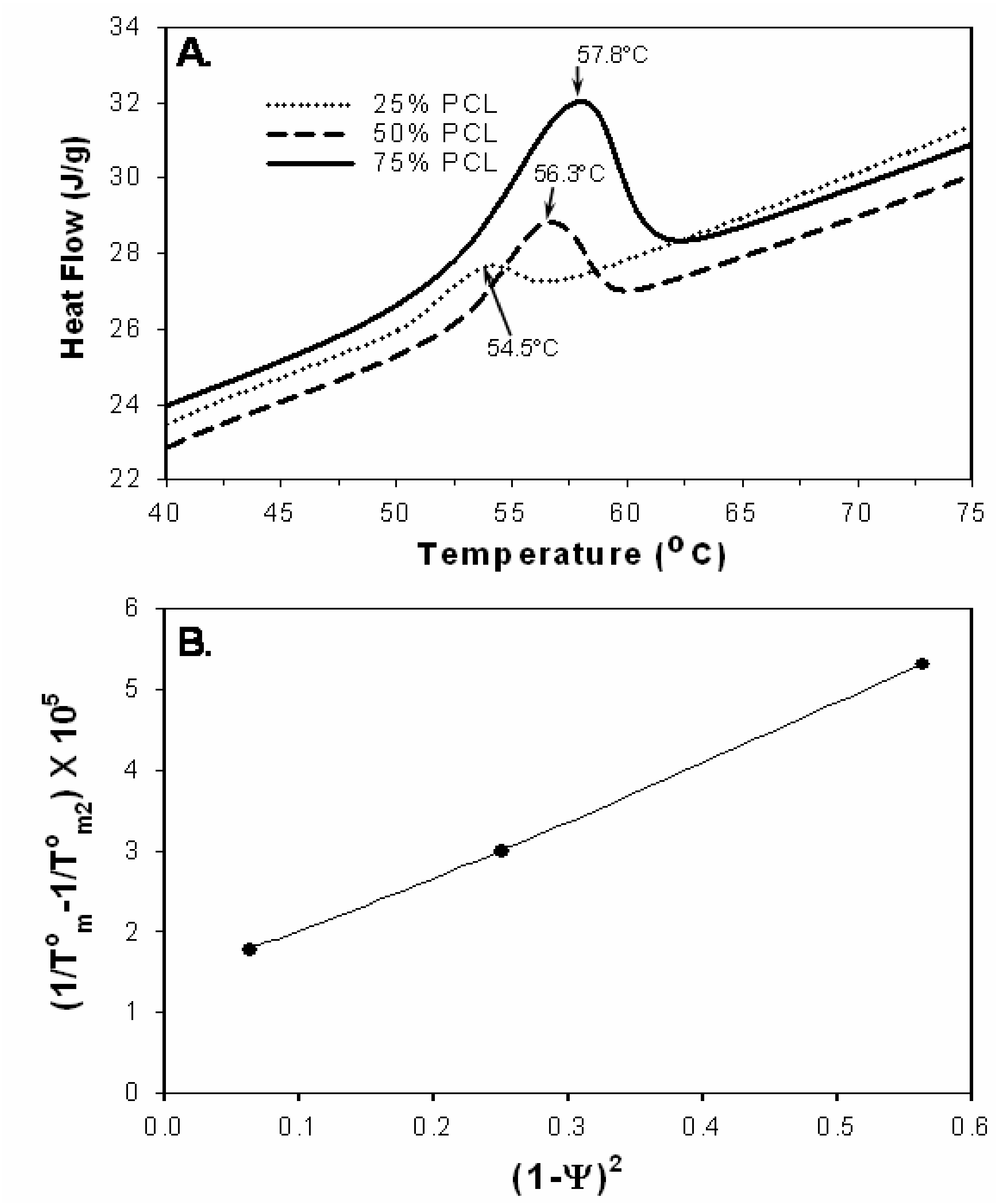

Figure 8.4. DSC thermograms of chitosan-PCL mixtures. A) Changes in melting point of PCL. B) Plot of Nishi-Wang equation to obtain Flory-Huggins interaction parameter (χ_{12}).

280°C, that corresponds to thermal decomposition which takes place before melting transition. The endothermic peak at 130°C maybe interpreted as the evaporation of residual water in the membranes. This peak does not signify the glass transition or melting of chitosan, as there no corresponding phase transition during cooling. Thermograms of mixtures showed a decrease in the T_m of PCL with increase in chitosan content (**Figure 8.4A**). This decrease could be due to the lowering of the chemical potential of the crystalline PCL in the mixtures. Flory- Huggins theory (Flory 1953) was used to better understand the interactions. Since the MW of both polymers are high, the simplified Nishi-Wang equation (Nishi T 1975) that neglects the terms containing degrees of polymerization was used to obtain the interaction parameter (χ_{12});

$$\frac{1}{T_m^0} - \frac{1}{T_{m2}^0} = \frac{-R}{\Delta H_{2u}} \frac{V_{2u}}{V_{1u}} \chi_{12} (1 - \Psi_2)^2$$

where subscripts 2 and 1 refer to PCL and chitosan respectively, $T°_m$ is the equilibrium melting temperature of PCL in mixtures (°K), $T°_{m2}$ is the equilibrium melting temperature of pure PCL (333.15°K), R is the universal gas constant (1.98 cal/mol.°K), ΔH_{2u} is the heat of fusion per mole of 100% crystalline PCL (3694.67 cal/mol) (Koenig and Huang 1995), V_{2u} and V_{1u} are molar volumes of the repeating units of the polymers (V_{2u} is 99.65 cm^3/mol, and V_{1u} is 1546.82 cm^3/mol) and ψ_2 represents volume fractions of PCL in the mixtures. To obtain χ_{12}, $\frac{1}{T_m^0} - \frac{1}{T_{m2}^0}$ was plotted against $(1 - \Psi_2)^2$ (**Figure 8.4B**). According to the equation, the line should pass through the origin. Since the line did not pass through the origin, it indicated that χ_{12} was composition-dependent. To find χ_{12} at each composition, lines were drawn from the origin to the corresponding points, and χ_{12} was calculated from the slope. The values of χ_{12} were -2.738, -3.479, and 8.274 for 25%, 50% and 75% PCL respectively. Negative values for interaction parameter χ_{12} indicate that the mixing reaction is exothermic which implies that chitosan and PCL are miscible.

8.3.4. Influence of mixing on dynamic mechanical and thermal properties: Chitosan undergoes thermal decomposition at 270°C prior to melting, and its T_g is unclear. On the other hand, PCL has a sharp T_m of 60°C, and T_g near -60°C. DMTA analysis showed the glass transition (α-relaxation) of PCL membranes formed from acetic acid at -35°C when it was heated from -100°C to 50°C (**Figure 8.5A**). There was no significant shift in the T_g of PCL in the mixtures indicating that the crystal structure of PCL may not be affected by the current method of mixing with chitosan. However, the damping loss (tan δ) associated with glass transition of PCL decreased with PCL content as evident from reduced height of the peaks. This suggests that the crystallinity of PCL is suppressed in the mixtures. Storage modulus (E') of chitosan (**Figure 8.5B**) was ten times higher than PCL due to its glassy/ amorphous nature. The hardness (E') of ductile PCL increased with the addition of brittle chitosan.

8.3.5. Influence of mixing on surface roughness and morphology: When the surface morphology of membranes was probed with AFM, three dimensional (3D) surface plots of height (**Figure 8.6**) showed that chitosan surface was smooth with uniformly distributed short spikes. Addition of PCL increased the height of these spikes. Seventy five percent PCL showed the tallest spikes over a large area whereas pure PCL was

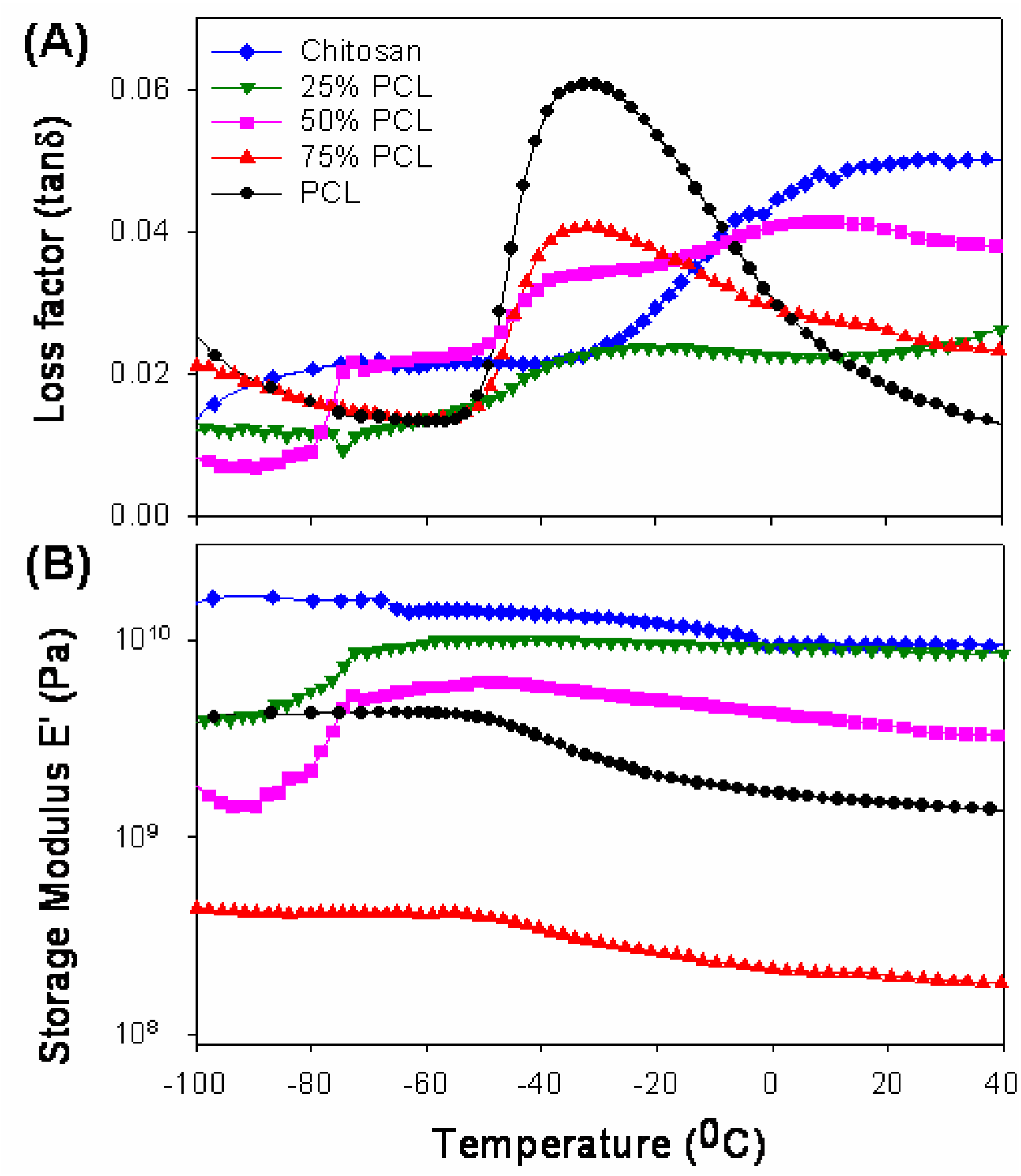

Figure 8.5. Influence of mixing chitosan and PCL on their dynamic thermo-mechanical properties. (A) Loss factor and (B) Storage modulus.

smoother than 75% PCL. Interestingly, preferential orientation of fibers was visible in 25% and 50% PCL, which was absent on pure membranes, suggesting that the presence of two components influences each other's vertical and horizontal alignment. When roughness was analyzed, a significant increase in roughness factors was observed in all the membranes containing PCL (**Figure 8.6**). Maximum roughness was observed in 75% PCL mixtures, which was significantly higher than 25% or 50% PCL mixtures.

8.3.6. Influence of mixing on surface charge density: EFM analysis of membranes gave 3D surface plots of vibrational frequency of cantilever (**Figure 8.7**). Chitosan showed a frequency shift of about 10 Hz, suggesting that has a net positive charge. Interestingly, PCL also showed a frequency shift of about 20Hz, suggesting that there is a positive charge on the surface. Given that PCL is an uncharged polymer, it maybe suggested that there is an induced dipole moment on PCL membranes. Among the blends, 25% PCL showed the maximum frequency shift (approximately 25Hz), followed by 50% and 75% PCL. As the frequency shifts in the composites are more than that in individual polymers, it maybe suggested that there is an interaction between cationic chitosan and the dipole-induced charge of PCL, which could affect the overall charge. In the images of the blends, the brighter domains might be related to PCL-rich phase, and the darker domains related to chitosan-rich phase.

8.4. DISCUSSION

Chitosan is a poly(1,4 D-glucosamine) molecule with amine functional group, which protonates in weak acids, and provides chitosan its unique cationic nature. Analysis by

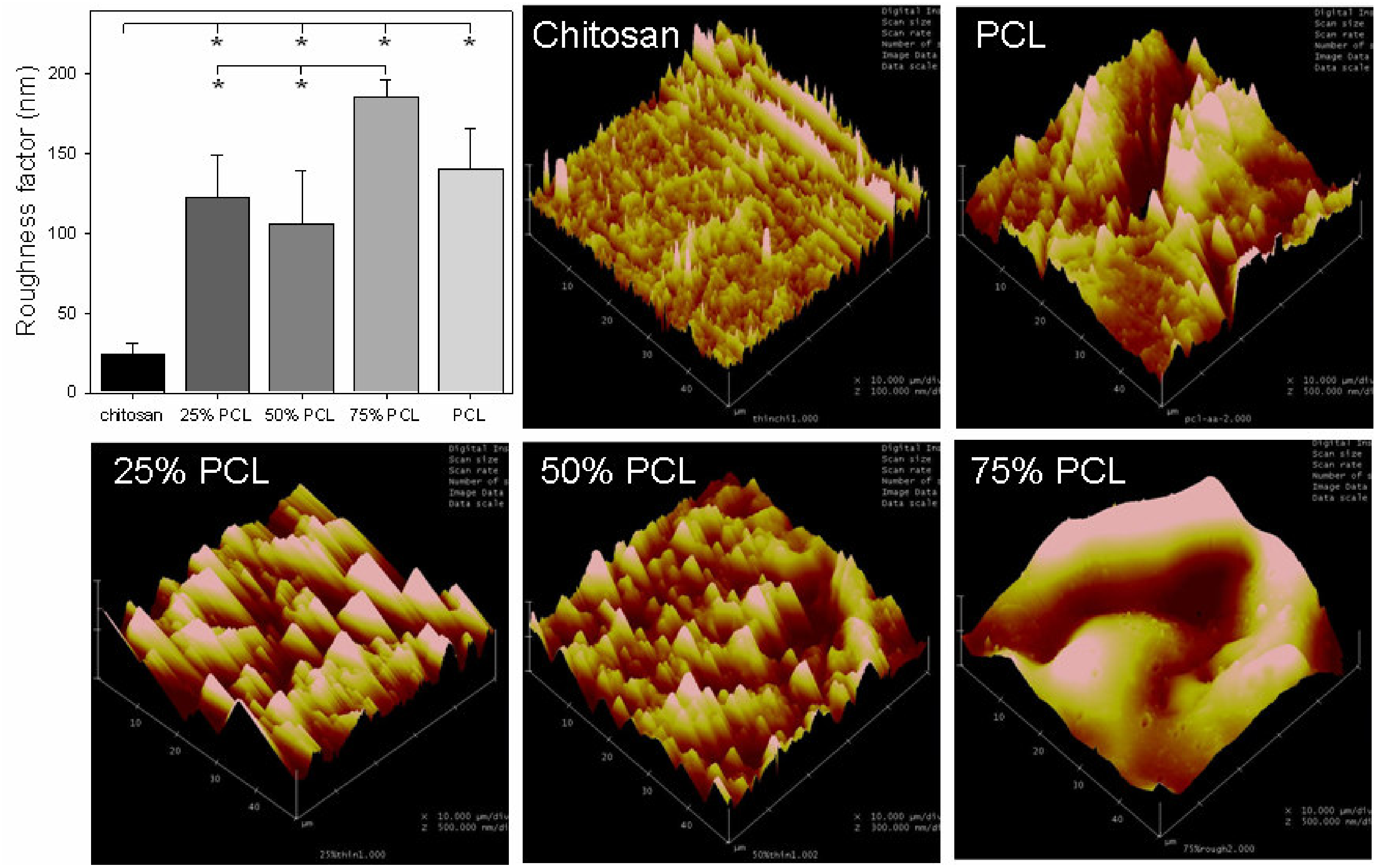

Figure 8.6. Influence of mixing chitosan and PCL on surface morphology of membranes by AFM. Bar graphs of roughness factors (*P<0.05 between the indicated groups) and representative AFM height images.

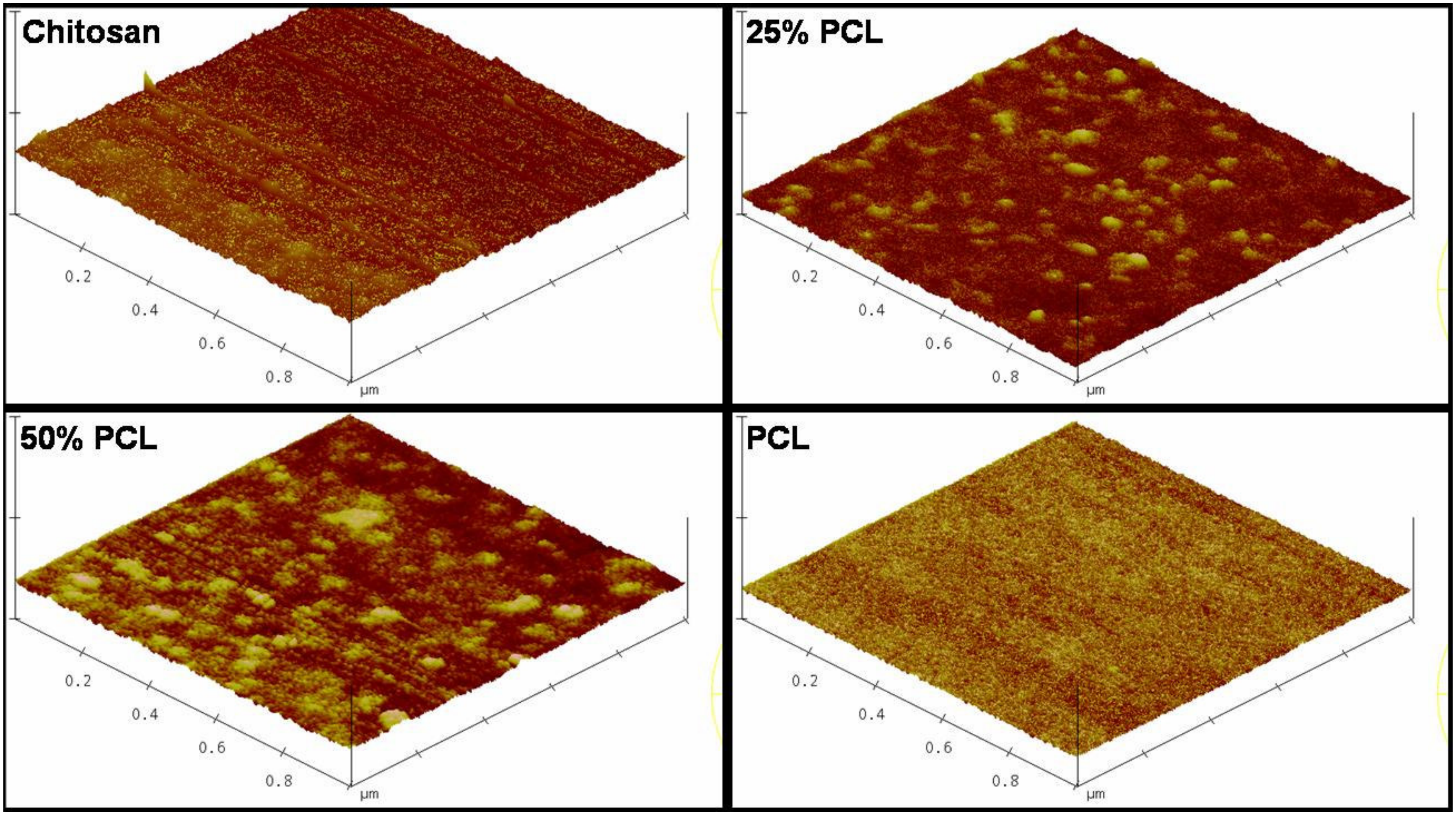

Figure 8.7. Influence of mixing chitosan and PCL on surface charge distribution of membranes by EFM. Frequency images of data scale 30Hz/div.

FTIR indicated no new chemical bonds in the mixtures, suggesting that there was no chemical reaction between the two polymers, which is consistent with literature reports of PCL mixtures with other polysaccharides (Cascone, Barbani et al. 2001). Other possible interactions include inter-molecular hydrogen bonding between oxygen atom of carbonyl group of PCL and hydrogen of hydroxyl group or ammonium ion of chitosan but they are not detectable by FTIR. Further studies are required to understand such interactions. Nevertheless, when the crystal structures were evaluated using WAXD, no differences were observed in the two polymers suggesting that chitosan and PCL co-exist as separate phases in the mixtures.

Chitosan and PCL provide a model system for studying miscibility of two semi-crystalline polymers, which is highly unpredictable. As chitosan undergoes thermal degradation prior to melting, the T_m of PCL was monitored to understand the miscibility of these two polymers. DSC studies showed a decrease in T_m with decrease in PCL content in the mixtures (Sarasam and Madihally 2005). Values of Flory-Huggins interaction parameter showed a concentration dependency, and partial miscibility of crystalline phase with amorphous phase. Since the depression in T_m of PCL could be due to the non crystalline phase acting as a diluent rather than altered crystal structure, this study evaluated the variations in T_g of PCL. First, no shift in the T_g of PCL in the mixtures reinforced that the PCL 'crystal structure' did not change. Secondly, peak area under the T_g of PCL reduced with increasing diluent composition confirming that PCL crystallinity was suppressed by chitosan, similar to other polyester- polysaccharide mixtures (Cascone, Barbani et al. 2001; Senda, He et al. 2002). However, these transitions were independent of each other without overlap in the relaxations confirming the findings from FTIR analysis that there was no chemical bonding between chitosan and PCL in the prepared mixtures.

Analysis of surface topographies and roughness factors indicated that the mixture surfaces were significantly rougher at the nanoscale than pure polymers. EFM analysis also suggested that the net positive charge of composite is higher than chitosan or PCL. As there were no chemical interactions between chitosan and PCL, or any significant changes in their physicochemical properties, the observed increase in biological activity of composites could be partially attributed to the increased surface roughness and charge density.

8.5. CONCLUSION

There was no detectable chemical reaction between chitosan and PCL in the composites. There were no changes in the crystal structure either. However, the crystallinity of the mixtures was reduced, due to partial interaction between chitosan and PCL. Composite surfaces showed increased roughness and positive charge, which might have influenced biological activity.

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CHAPTER IX

CONCLUSION AND FUTURE DIRECTIONS

9.1. CONCLUSION

The objective of this thesis was to develop a novel biomaterial with superior mechanical and biological properties, aimed at tissue engineering applications. The approach taken to achieve this goal was to blend a natural polysaccharide chitosan, with a synthetic polyester polycaprolactone (PCL). Based on the results obtained, the conclusions with regards to the specific aims of the project are as follows:

9.1.1. Obtaining a homogeneous mixture of chitosan and PCL.

Solvent dissolution was chosen over chemical modifications to combine chitosan and PCL, in order to preserve their individual properties in the composites. The absence of a common solvent to dissolve hydrophilic chitosan and hydrophobic PCL was resolved by formulating a unique but simple 77% acetic acid solution. Unlike hexafluoroisopropanol, which others have used as a common solvent, this novel concoction is non-toxic. Mixture solutions were homogenous and the relative mass ratios of chitosan and PCL could be easily varied, to a certain extent.

Several conditions were evaluated to optimize the formation of 2D membranes and 3D scaffolds. Uniform membranes of mixtures were fabricated by drying the solutions in an oven at 55°C. Porous scaffolds were obtained by freeze-drying technique by dissolution in 25% acetic acid plus 0.5mL of chloroform.

9.1.2. Biomechanical and degradation properties of chitosan-PCL mixtures *in vitro*.

Chitosan/PCL mixture membranes showed tensile properties (strength and elongation at break) that were comparable to chitosan. These properties are alterable by changing the processing conditions and composition. Drying the solutions at 55°C in oven and annealing with chloroform gave superior mechanical properties to the composites. 50% PCL had better tensile properties, compared to the other mixtures. Performance under cyclical loading is reported for the first time in this research. Within the elastic region, chitosan, PCL, and their composite membranes were able to sustain 10 cycles of load, without break.

Composite membranes of chitosan and PCL showed no significant weight loss over 4 week period of study, even in the presence of lysozyme. This implies that the degradation rate of chitosan membranes was not altered by mixing with PCL. Therefore, alternative methods such as scaffold design and lowering the MWs of chitosan and PCL should be explored to enhance the degradation rate of composites.

The combination of chitosan and PCL in the presence of 77% acetic acid did not cause cytotoxicity in 2D or 3D form. Mixture membranes showed improved viability and spreading of mouse embryonic fibroblasts, compared to control or individual polymers. Chorioallantoic membrane analysis suggested that 3D porous scaffolds were biocompatible and their morphology showed better support for the formation of vasculature, compared to pure polymers.

Chitosan/PCL composites showed increased support for biological activity of bacteria also. Adherence and proliferation of Gram-positive *Streptococcus mutans* and Gram-negative *Actinobacillus actinomycetemcomitans* bacteria was higher on the mixtures, relative to chitosan or PCL. In other words, the antibacterial nature of chitosan was compromised in the presence of PCL. This research also showed that the antibacterial properties of chitosan were contact-dependent and more potent against Gram-positive bacteria, compared to Gram-negative bacteria.

9.1.3. Interaction between chitosan and PCL in the mixtures and changes in their physicochemical properties.

When chitosan and PCL were dissolved in 77% acetic acid, there was no chemical reaction between them, as detected by FTIR. There was no change in their crystal structure either, suggesting that chitosan and PCL co-existed in the mixtures. However, crystallinity of these polymers was reduced in the mixtures suggesting partial miscibility between chitosan and PCL. This could be due to hydrogen bonding between amine/ hydroxyl functional groups of chitosan and carbonyl group of PCL. Further analysis is required to confirm this interaction.

9.1.4. Influence of mixing and surface characteristics on biomechanical properties.

Chitosan and PCL composite membranes showed increased roughness and positive charge density on the surface. As there were no physiochemical changes in the mixtures, observed changes in biological activity of chitosan could be attributed to surface morphology. Therefore, it maybe concluded that rougher surfaces and higher positive charge favors cellular colonization of both prokaryotes and eukaryotes.

9.2. FUTURE DIRECTIONS

In lieu of the results obtained and the above stated conclusions, the following recommendations are made for further improvement, processing and characterization of chitosan/PCL composites.

9.2.1. Evaluation of surface hydrophilicity of mixture membranes and their influence on cellular activity: The observed increase in biological activity of mixtures relative to chitosan was attributed to surface roughness and positive charge density, due to absence of other physicochemical changes.

One of the reasons why PCL does not exhibit bioregulatory activity is its hydrophobicity. On the other hand, chitosan is highly hydrophilic. When these two polymers are mixed in 77% aqueous acetic acid solution, significant alterations in the hydrophilicity of the mixtures might occur. Such alterations might also be the reason for increased viability of cells on 50% and 75% PCL mixtures. Therefore, the hydrophilicity of the prepared mixture membranes needs to be measured by contact angle method.

Contact angle experiments give information about the interaction energy at the interface of a solid surface and liquid (Degennes 1985). When water is used, larger contact angle indicates more hydrophobicity of the surface, and vice versa. However, when measuring chitosan/PCL composite membranes, the variations in surface charge density and roughness need to be taken into account as electrostatic interactions between the surface and liquid, as well as topographical differences could influence the interfacial interaction energy.

9.2.2. Further improvement of biomechanical properties of chitosan/PCL composites: In this project, 77% acetic acid was used to dissolve PCL and chitosan in different ratios. By further reducing the amount of water phase, the mechanical properties of pure PCL in acetic acid could be achieved. This can be done by adding 1.5mL of 2% chitosan to 10mL of acetic acid containing PCL, instead of 3mL of 1% chitosan. If low MW chitosan is used, the water content could be further reduced by adding 1mL of 3% chitosan to 10mL of acetic acid-PCL solutions.

Adding water to PCL- acetic acid solutions first, followed by dissolution of calculated amounts of chitosan might also give a pure PCL-like membrane, instead of adding chitosan solution to PCL-acetic acid solutions.

The biological properties of chitosan/PCL composites need to be further explored. Detailed studies regarding cell differentiation, growth kinetics, absorption and deposition of ECM components on these mixtures are required. Other bioactive molecules such as GAGs could be immobilized on these matrices for specialized cellular responses. For example, heparin, which acts as an anti-coagulant could be conjugated onto these matrices to improve their hemocompatibility (Wang, Li et al. 2003).

9.2.3. Formation of porous scaffolds: Using 77% acetic acid, chitosan and PCL were homogenously mixed in different mass ratios and processed into stable and uniform membranes. However, limited success was achieved in obtaining porous scaffolds from mixtures. Sturdy and stable three dimensional porous structures of pure chitosan and PCL can be obtained by freeze-drying and freeze-extraction processes, respectively. However, mixture scaffolds formed by freeze-drying were dimensionally unstable due to large amounts of acetic acid present in the solution. Using mixtures with higher polymer concentrations and molecular weights might give better scaffolds by freeze-extraction process.

Mixture scaffolds could not be formed by freeze-extraction method, as adding acetone of -20°C temperature thawed and dissolved the scaffolds frozen at -80°C. This thermal transition can be minimized by adding acetone precooled to -80°C for extraction. As the mixture solutions do not freeze at -20°C, and form unstable scaffolds at -80°C, freezing at a lower temperature such as -190°C (by dipping in liquid nitrogen) may form close-knit and strong scaffolds. Lower freezing temperatures tend to form a tighter network by creating smaller pores (Moshfeghian 2005).

If the above alternatives do not work, freeform fabrication of scaffolds is recommended for their high accuracy in design and fabrication of 3D structures, ease of use, and time and energy saving efficiency (Leong, Cheah et al. 2003). This technology involves computer-aided design and controlled polymer deposition though a microvalve nozzle system. It gives better control over micro-architectural features of scaffold design, and even biological molecules could be integrated into the matrix during fabrication (Khalil and Sun 2006).

As with 2D membranes, the biomechanical and degradation properties of the formed scaffolds need to be evaluated. The influence of pore size, shape, and porosity on cellular colonization needs to be examined for chitosan/PCL composites, similar to chitosan/PLGA mixtures (Huang 2005).

9.2.4. Evaluation of biomechanical and degradation properties in dynamic physiological conditions: Recently, it was shown that high shear stresses reduced cell spreading and adhesive strength on chitosan based matrices, under simulated dynamic physiological conditions (Huang 2005), dispelling previously held myths that chitosan supports cellular adhesion and spreading. In lieu of these findings, the results obtained with chitosan/PCL composites in this project should be further clarified by conducting biomechanical studies in a bioreactor, designed to simulate shear stresses inside the body.

Improved biological properties of the composites were attributed to surface morphological features such as roughness and charge distribution on 2D membranes. However**,** the morphology and surface features were evaluated in a static environment i.e. in a dry state, at room temperature. These parameters need to be evaluated in a hydrated state at 37°C with and without serum proteins.

9.2.5. Evaluation of biomechanical properties of mixtures *in vivo*: In this project, chitosan and PCL mixtures showed improved biocompatibility in terms of cellular viability and spreading in 2D form, and better support for vasculature in 3D form. However, these results are obtained *in vitro* under simulated physiological conditions, and cannot be directly applied to *in vivo*. In biomedical applications, *in vitro* studies only give a preliminary estimate of the material's feasibility. The actual performance and success of a material may vary significantly *in vivo* where a number of other physical, chemical, metabolic, and immunological factors exert a simultaneous influence. Therefore the performance of the prepared biomaterials needs to be tested *in vivo* using animal models.

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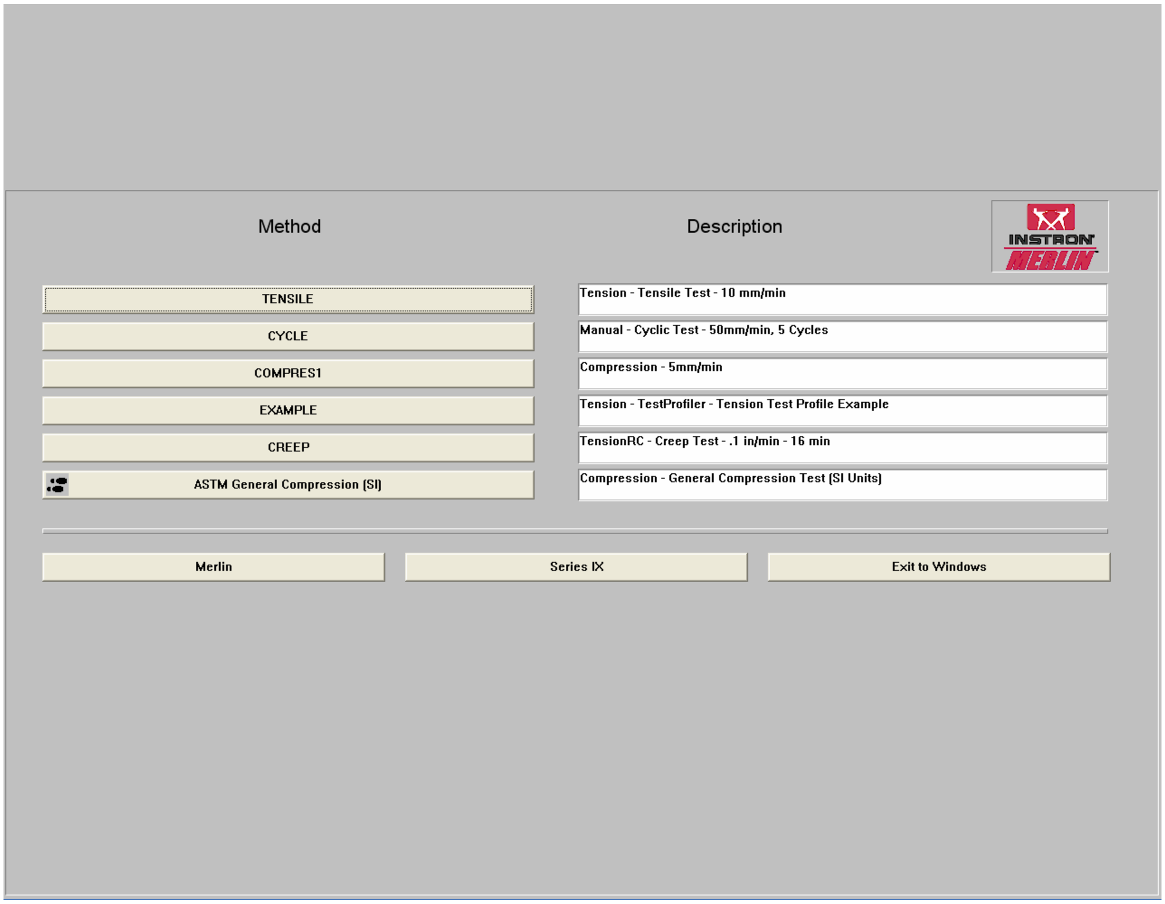
APPENDIX

ILLUSTRATION OF MECHANICAL TESTING USING INSTRON 5542

1. Turn on the power switch, which is located at the back of the Instron machine and open MERLIN software in the computer. Close all other programs.

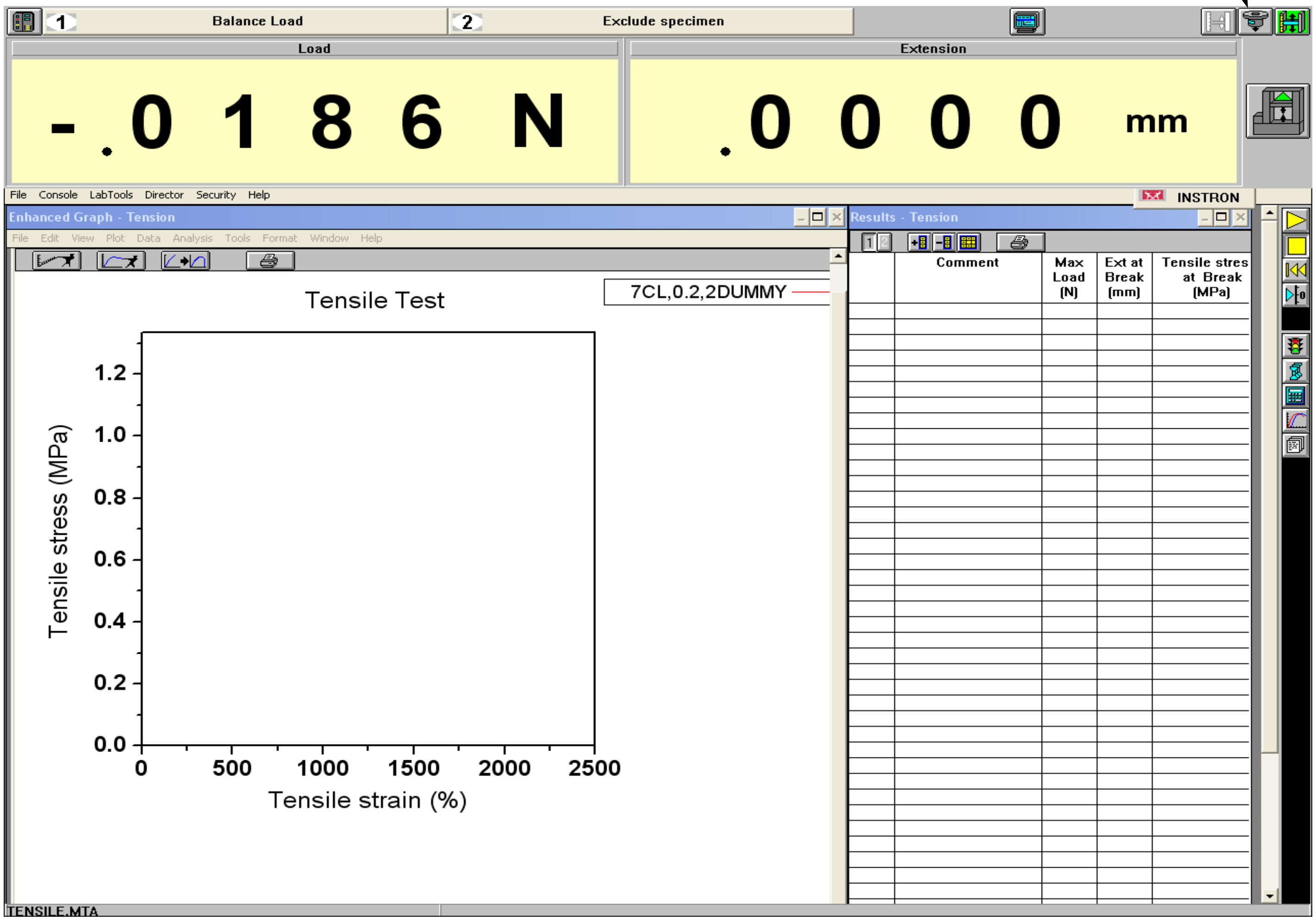
2. The following screen appears showing the six most recently used tests. Click on the desired test or click on MERLIN and select method from TEST TYPE for more options.



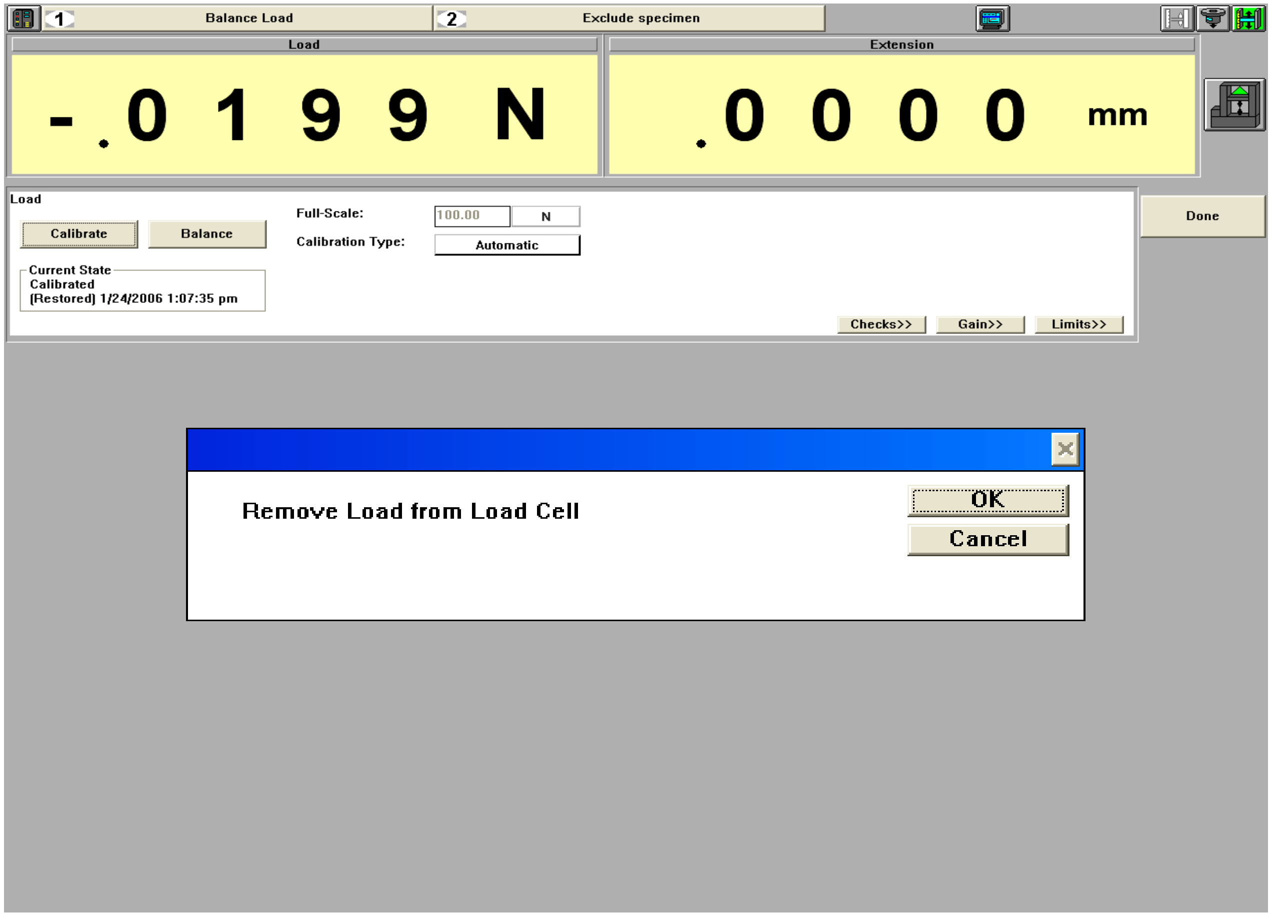
Method	Description
TENSILE	Tension - Tensile Test - 10 mm/min
CYCLE	Manual - Cyclic Test - 50mm/min, 5 Cycles
COMPRES1	Compression - 5mm/min
EXAMPLE	Tension - TestProfiler - Tension Test Profile Example
CREEP	TensionRC - Creep Test - .1 in/min - 16 min
ASTM General Compression (SI)	Compression - General Compression Test (SI Units)

3. Whichever method you select, the following screen appears. Click on the LOAD CELL icon to calibrate the system (required only once in a day).

Load cell



4. The following screen appears. Click CALIBRATE. A warning appears to remove load from load cell. Make sure that top grip is free and click ok.



-.0199 N

.0000 mm

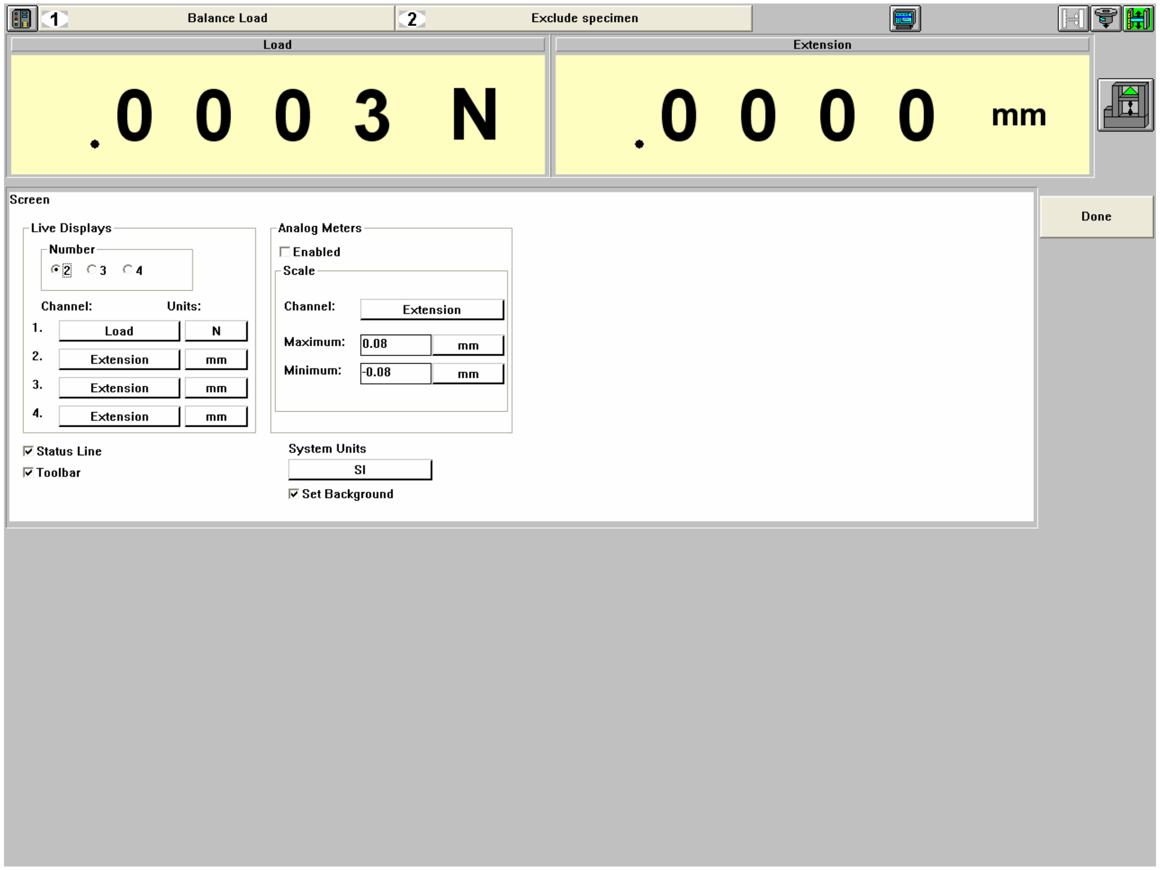
Full-Scale: 100.00 N

Calibration Type: Automatic

Current State
Calibrated
(Restored) 1/24/2006 1:07:35 pm

Remove Load from Load Cell

5. After calibration, click done to return to the main screen. From this page, you can also select the number of live displays you want and the corresponding units. For example, in this screen, I have selected two displays- load and extension in SI units. Click on DONE to return to main screen.



Load .0003 N

Extension .0000 mm

Screen

Live Displays

Number ● 2 ○ 3 ○ 4

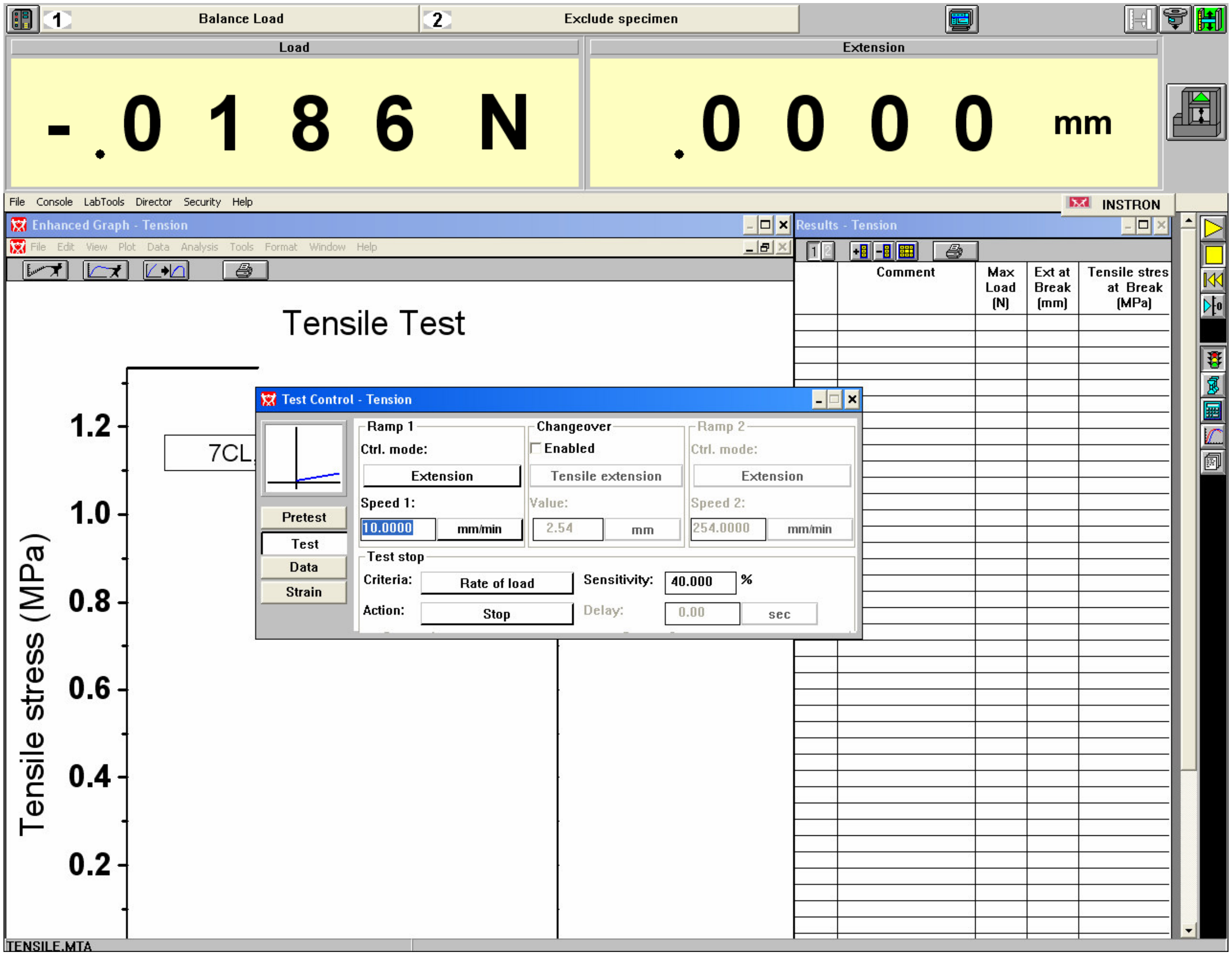
Channel:	Units:
1. Load	N
2. Extension	mm
3. Extension	mm
4. Extension	mm

☑ Status Line
☑ Toolbar

Analog Meters
☐ Enabled
Scale
Channel: Extension
Maximum: 0.08 mm
Minimum: -0.08 mm

System Units: SI
☑ Set Background

6. Back on the main screen, on the right side tool bar, there are icons to start test, stop test, return to gauge length and reset gauge length. These are actions which can also be performed using the keypad on the Instron machine. There are other icons for test control, sample parameters, results, graph and report. Click on TEST CONTROL and a small window appears. Here for tensile testing, I define my speed of extension (also called crosshead speed) as 10mm/min.



Load: -.0186 N

Extension: .0000 mm

Comment	Max Load (N)	Ext at Break (mm)	Tensile stres at Break (MPa)

Ramp 1

Ctrl. mode: Extension

Speed 1: 10.0000 mm/min

Changeover

☐ Enabled

Tensile extension

Value: 2.54 mm

Ramp 2

Ctrl. mode: Extension

Speed 2: 254.0000 mm/min

Test stop

Criteria: Rate of load

Sensitivity: 40.000 %

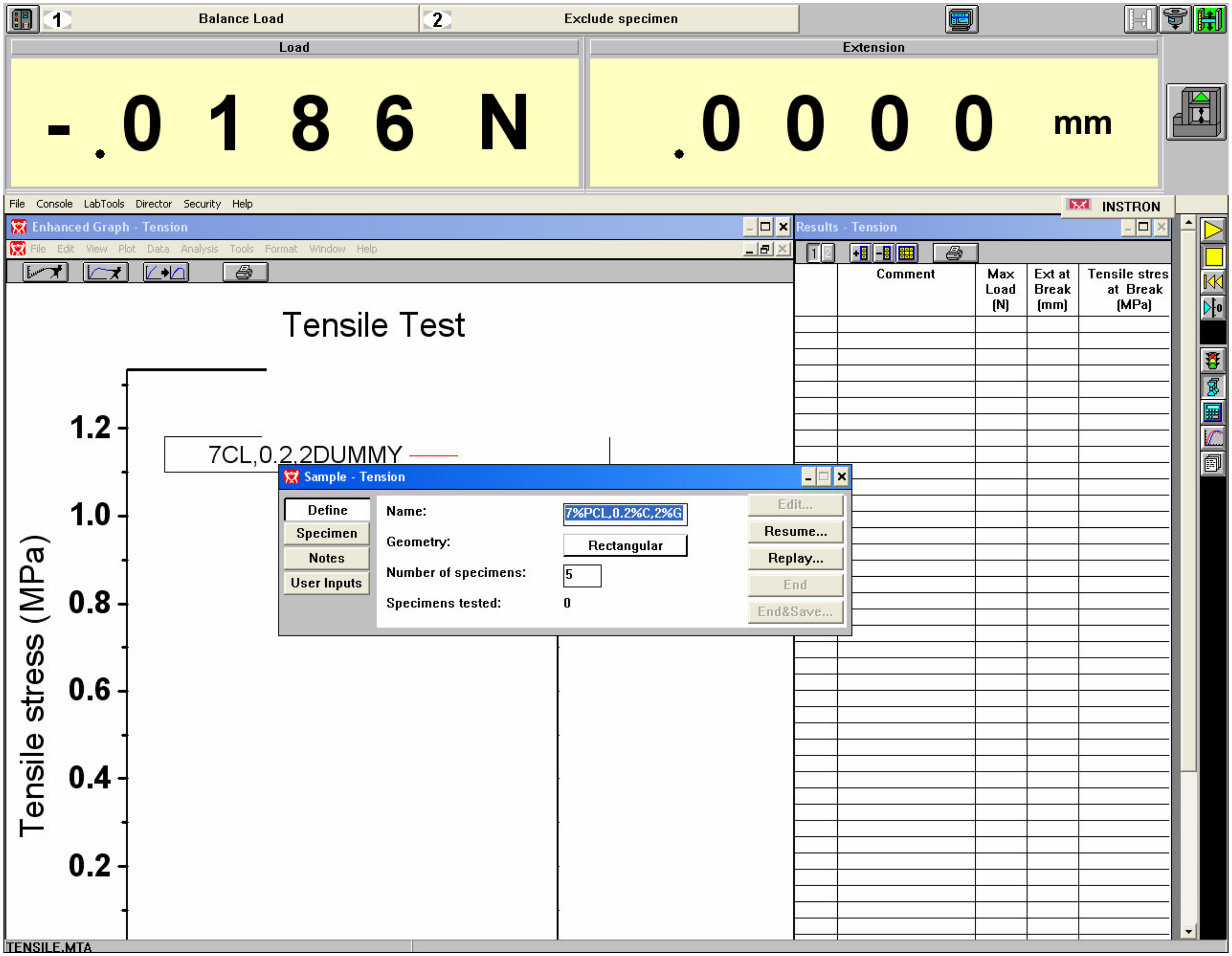
Action: Stop

Delay: 0.00 sec

7. Using the JOG UP and JOG DOWN buttons on the machine keypad and using forceps, insert the sample between the grips. For the first time, make sure the toggle switches behind the grips are closed (towards the center) and press the foot switch to close the grips. Second time onwards, you can just use the toggle switch to open and close the grips. Use the FINE POSITION button to stretch the sample properly after gripping.

8. Click RESET GUAGE LENGTH icon (or push corresponding button on keypad) and measure the distance between the grips which is the gauge length or the length of the sample being tested. Do not forget this step.

9. Close the test control window and open the SAMPLE PARAMETER window. You can define your sample and specify an approximate number of specimens that you are going to test. Select the appropriate geometry of the samples.



Load: -.0186 N

Extension: .0000 mm

Comment	Max Load (N)	Ext at Break (mm)	Tensile stres at Break (MPa)

Name: 7%PCL,0.2%C,2%G

Geometry: Rectangular

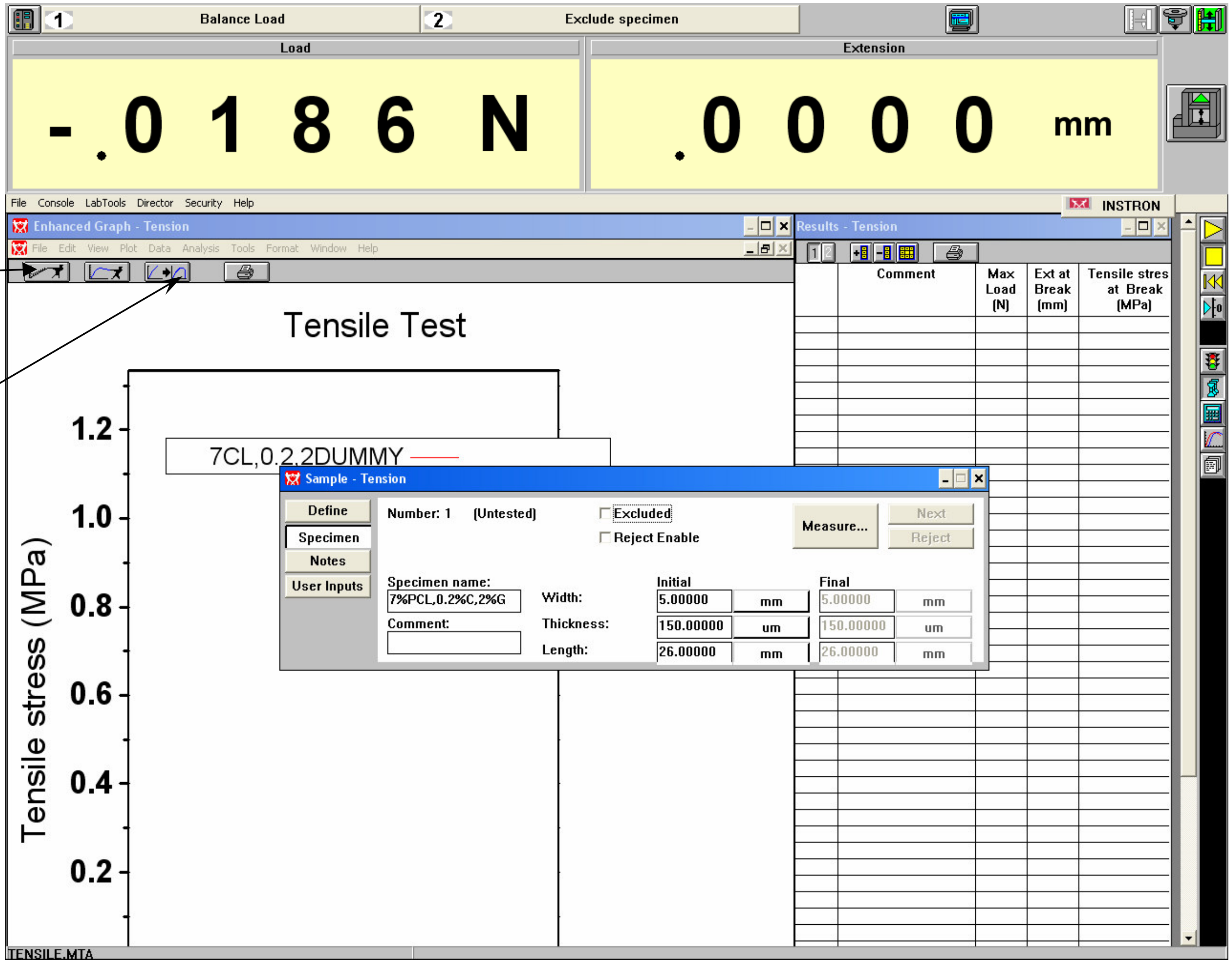
Number of specimens: 5

Specimens tested: 0

10. On the SPECIMEN tab, specify the specimen number or name, width, thickness and length with appropriate units. Close the SAMPLE window and click on START TEST. Test will stop automatically as per test specifications (break in tensile test). If it does not, you can manually STOP TEST. To test next specimen, click on NEXT in this window. If you want to change the dimensions of a specimen that has already been tested, go to define, edit and change the values for the respective specimen(s); the software will recalculate the data with the changed parameters.

Choose display axis and units

Refresh view while testing



Load: -.0186 N

Extension: .0000 mm

Comment	Max Load (N)	Ext at Break (mm)	Tensile stres at Break (MPa)

Number: 1 (Untested)

☐ Excluded

☐ Reject Enable

Specimen name: 7%PCL,0.2%C,2%G

Comment:

	Initial		Final	
Width:	5.00000	mm	5.00000	mm
Thickness:	150.00000	um	150.00000	um
Length:	26.00000	mm	26.00000	mm

11. The results are calculated and displayed in the spread sheet. The displays (eg. stress, strain, yield, modulus) can be selected from the setup on results menu. If you are interested only in ASCII data, you can close the results window. The graph options can be accessed using the icons and the menu in graph window.

12. To end test, click FILE-DATA-END AND SAVE. Select a location and name to save the data. ASCII data will be stored in C: INSTRON/USER/DATA in the form a '.raw' file. Open these files with Microsoft excel, select first column, and click Data- text to columns- next- check the comma option- finish. Save as Excel workbook. This way, you can plot any axes and specimens against each other.

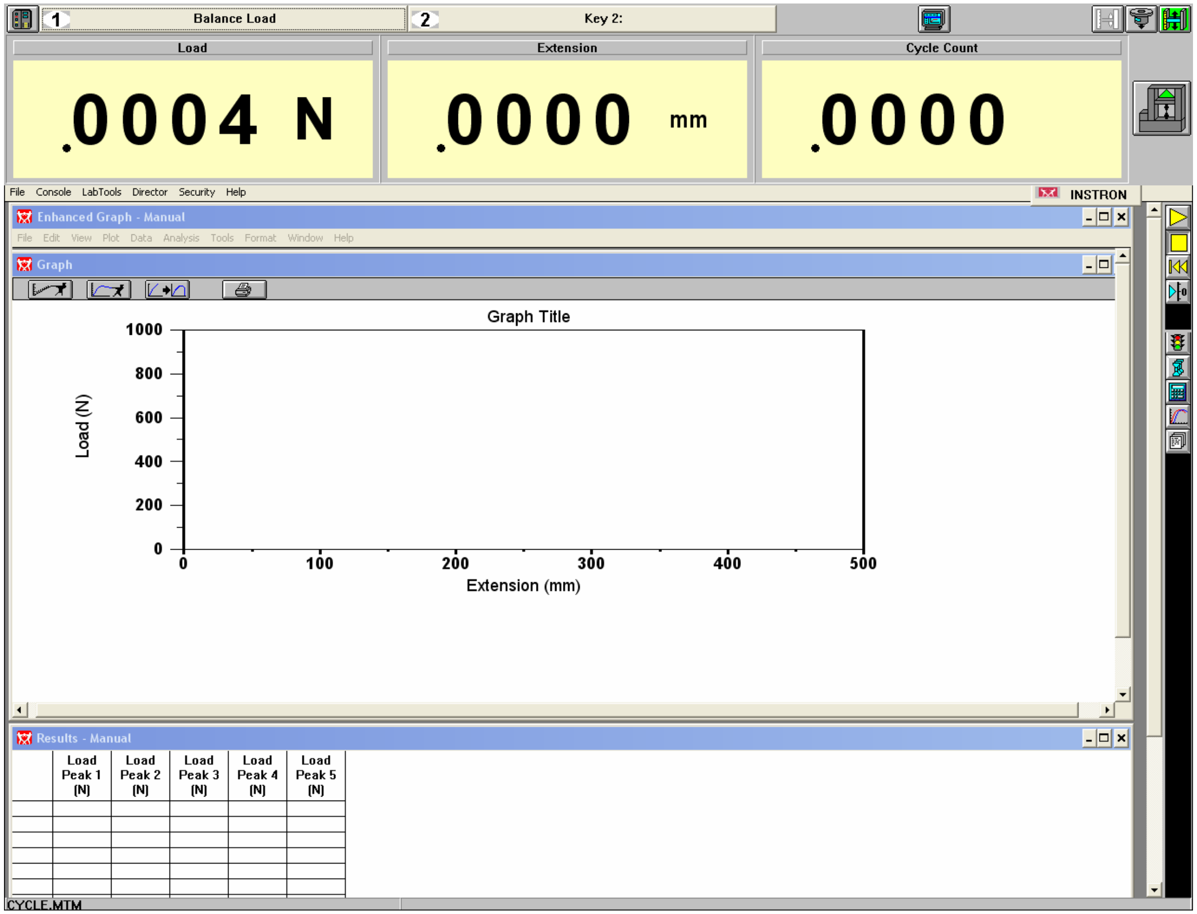
13. To resume a test of the same test, after calibration, go to FILE- DATA- RESUME and select the desired file to run. If you want to change parameters of a sample that has already been tested and saved, go to FILE- DATA- REPLAY, select the desired file and input the changes.

14. To close software, click FILE-EXIT. A warning to save method will appear; you can save any changes that you made to the test or for custom designed tests, otherwise ignore this warning. Turn off the machine at the back.

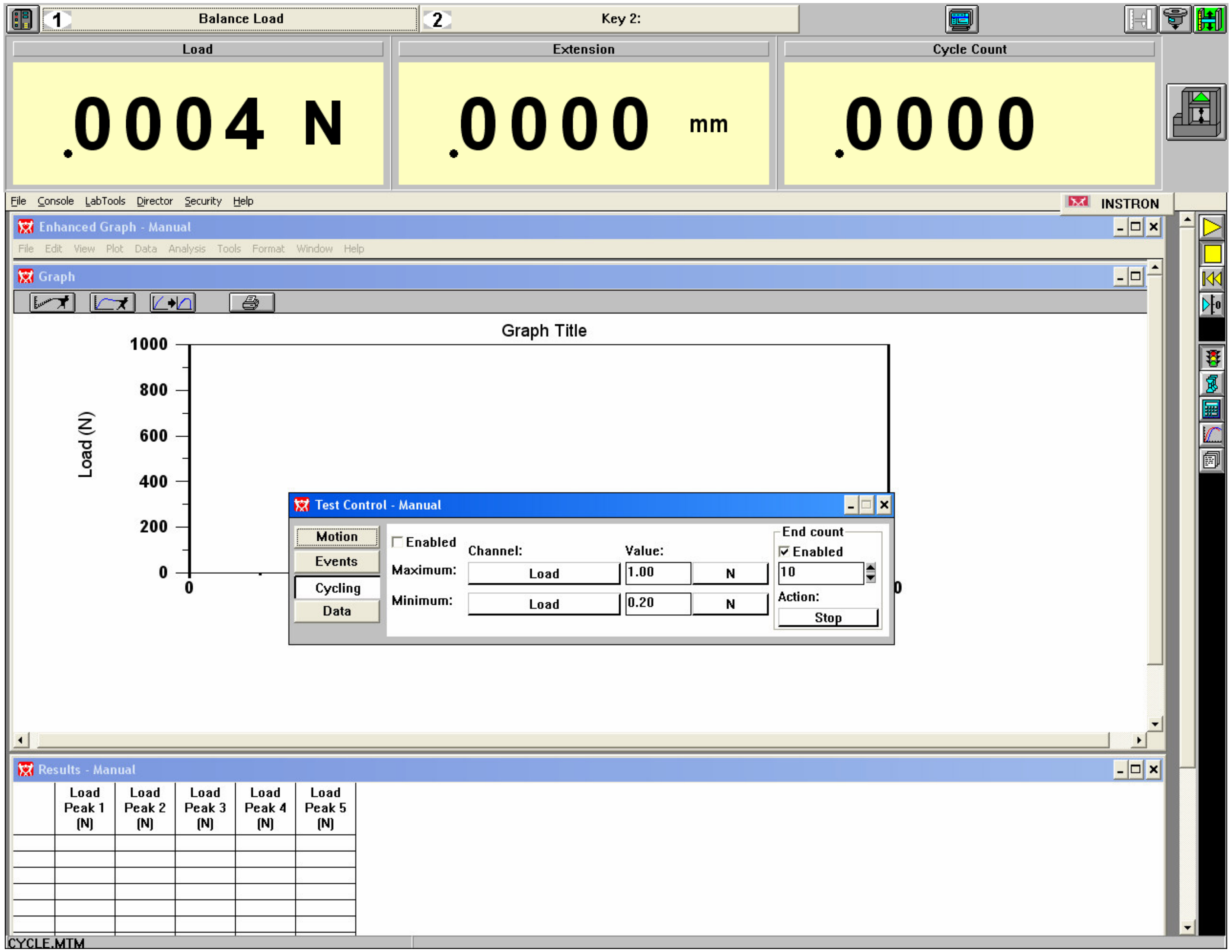
15. If the system shuts down due to some problem, data will be saved under C: INSTRON:USER:DATA under a default name after some time. These files can be resumed.

16. If CYCLE test method is chosen, the screen looks as follows as I have setup an additional display to show the number of cycles.

17. The test method is defined under the TEST CONTROL- CYCLING, where the load limits of the elastic region of a sample (these are determined by a regular tensile test) and arbitrary number of cycles is specified. Check ‘enabled’ and close the window and start the test.



	Load Peak 1 (N)	Load Peak 2 (N)	Load Peak 3 (N)	Load Peak 4 (N)	Load Peak 5 (N)



Load: .0004 N

Extension: .0000 mm

Cycle Count: .0000

Test Control - Manual (Cycling)

☐ Enabled

	Channel:	Value:	
Maximum:	Load	1.00	N
Minimum:	Load	0.20	N

End count

☑ Enabled

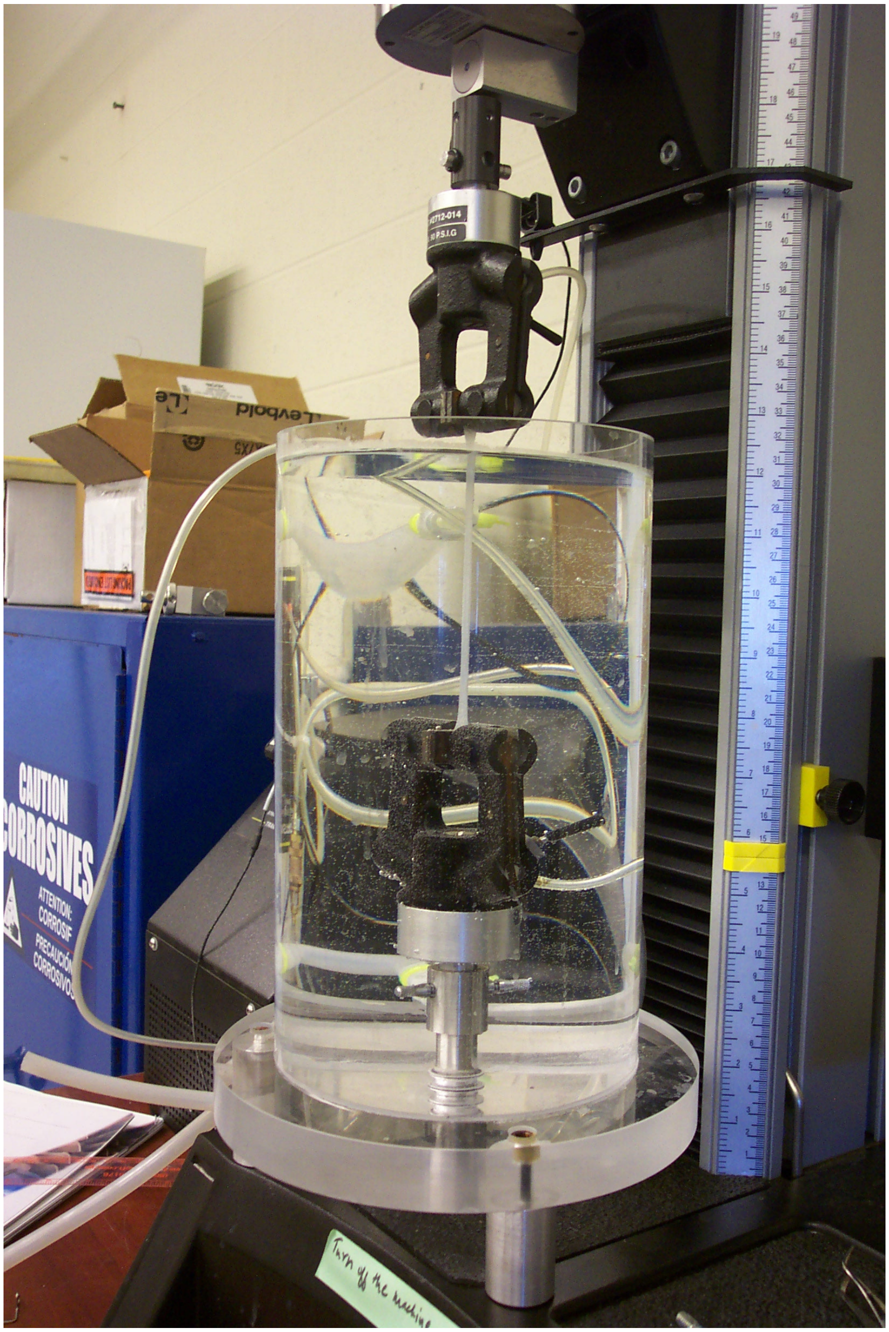
10

Action: Stop

	Load Peak 1 (N)	Load Peak 2 (N)	Load Peak 3 (N)	Load Peak 4 (N)	Load Peak 5 (N)

CYCLE.MTM

18. For testing at wet 37°C, samples are gripped and submerged in Phosphate Buffer Saline (PBS) during testing in a custom-built environmental chamber. The PBS was maintained at 37°C in a bath and continuously circulated through the chamber using a peristaltic pump, with an inlet pipe at the bottom of the chamber and an outlet pipe at the top.



VITA

Aparna Reddy Sarasam

Candidate for the Degree of

Doctor of Philosophy

Thesis: CHITOSAN- POLYCAPROLACTONE MIXTURES AS BIOMATERIALS- INFLUENCE OF SURFACE MORPHOLOGY ON CELLULAR ACTIVITY

Major Field: Chemical Engineering

Biographical:

Personal Data: Born to S. Gnyaneshwar Reddy and Suvarna on August 12th 1979 in Hyderabad, India

Education: Obtained secondary school certificate in 1994 from St. Anthony's High School, high school certificate in 1996 from St. Ann's Junior College and graduated with a Bachelor of Technology in Chemical Engineering certificate (1997-2001) from Indira Seva Sadan Engineering College (Affiliated to Jawaharlal Nehru Technlogical University), all located in Hyderabad. Completed the requirements for the degree of Doctor of Philosophy, with a major in Chemical Engineering at Oklahoma State University in July' 2006.

Experience: Worked as a teaching assistant for undergraduate level courses in Engineering Thermodynamics (ENSC 2213) and Introduction to Chemical Engineering (ChE 2033) at Oklahoma State University from January' 2002 to May' 2004. Worked as a Research Assistant in School of Chemical Engineering at Oklahoma State University from May' 2002 to July' 2006

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Title of Study: CHITOSAN- POLYCAPROLACTONE MIXTURES AS BIOMATERIALS- INFLUENCE OF SURFACE MORPHOLOGY ON CELLULAR ACTIVITY

Pages in Study: 157 Candidate for the Degree of Doctor of Philosophy

Major Field: Chemical Engineering

Scope and Method of Study: Chitosan, a naturally occurring polysaccharide has excellent biocompatibility, positive charge and reactive functional groups that help regulate cellular activity, antibacterial properties, and low cost. However, inadequate mechanical strength and incomplete understanding of cell-material interactions have limited its success. Polycaprolactone (PCL) is synthetic polyester that is easy to process, with excellent mechanical properties and tailorable physicochemical properties but lacks bioregulatory properties. Chitosan and PCL were homogenously dissolved in a unique acetic-acid water mixture to obtain a composite that has good biomechanical properties. Uniform membranes were obtained by drying the solutions at 50°C and several processes were explored for forming porous scaffolds, including freeze-extraction and freeze-drying. Membranes were tested for tensile properties under dry/wet, and monotonic/cyclical loading conditions. Support for adhesion and growth of eukaryotes (mouse embryonic fibroblasts) and prokaryotes (*Streptococcus mutans* and *Actinobacillus actinomycetemcomitans*) were evaluated *in vitro*. Cytocompatibility and support for vasculature of scaffolds was tested using CAM assay. Degradation characteristics were evaluated in PBS over 4 weeks. Physicochemical properties and interactions in these mixtures were characterized by DSC, DMTA, FTIR, WAXD, SEM, AFM and EFM.

Findings and Conclusions: Hydrophilic chitosan and hydrophobic PCL were successfully mixed in different mass ratios by dissolution in a unique water-acetic acid mixture. There was no chemical reaction between chitosan and PCL in the prepared mixtures. However, the crystallinity was reduced suggesting partial miscibility between these polymers. Tensile properties of mixture membranes were comparable or better than those of chitosan membranes. These membranes also showed improved viability and spreading of mouse embryonic fibroblasts as well as Gram-positive and Gram-negative bacteria. 3D scaffolds were non-toxic and supported blood vessel formation on CAMs. Evaluation of surface showed increased roughness and positive charge density on the mixtures. Observed changes in biological activity of chitosan were attributed to surface characteristics due to absence of other physical or chemical changes. Therefore, it maybe concluded that higher roughness and cationic charge density favor cellular colonization.

ADVISER'S APPROVAL: Dr. Sundararajan V. Madihally