

**Technical University of Liberec**

**Faculty of Mechatronics, Informatics and Interdisciplinary Studies**



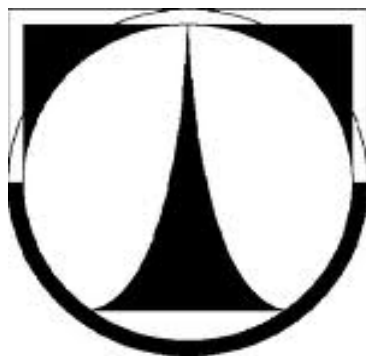
# **Diploma Thesis**

**2010**

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**Technická Univerzita v Liberci**

**Fakulta mechatroniky, informatiky a mezioborových studií**



# **Diplomová Práce**

**2010**

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Technical University of Liberec

Faculty of Mechatronics, Informatics and Interdisciplinary Studies

Department of Novel Technologies and Applied Informatics

## ELECTROSPUN STARCH COMPOUNDED POLYMER SCAFFOLDS

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## ***Abstract***

Electrospinning has been widely known during decades ago as a unique technique to manufacture fibrous mats. The focus of the electrospinning process in tissue engineering is on generating three dimensional matrix scaffolds which can ultimately be used to promote primary cell activities starting from cell attachment and proliferation, as well as complex processes such as differentiation which is completely advantageous, since the resultant fibers seemingly mimic the fibrillar native tissue structures. In this work, a variety of biopolymers such as poly ( $\epsilon$ -caprolactone) PCL, starch acetate blended PCL, starch acetate coated PCL were proposed as promising candidates for tissue scaffolding. In essence, the main goals of this works are to generate three dimensionally matrix scaffolds from such polymer scaffolds by electrospinning, thereafter the electrospun fibrous mats made up of starch based scaffolds could promote cell viability demonstrated by an MTT assay. Results of the current investigation demonstrated that the chondrocyte cells attached on the starch acetate blended PCL surface showed slightly higher in cell viability compared to polymer based scaffold, PCL. Another type of polymer scaffolds, starch acetate coated PCL demonstrated the defined cells were much more stretched and spread similar to PCL-based scaffold. Thus, starch ester and its blended forms (starch acetate blended and coated PCL) could be potentially utilized to replace PCL as a promising candidate for the scaffolds in tissue engineering since the results of MTT assay given did not significantly alter among a pristine synthetic polymer scaffold, but other challenging efforts in the future will obviously be required to improve their characters to be similar to the widely used control scaffold, Tissue Control Polystyrene (TCP).

## *Abstrakt*

Elektrostatické zvlákňování je známo, od počátku minulého století jako unikátní metoda k výrobě vláknenných vrstev. Využití elektrostatického procesu pro tkáňové inženýrství je založena tvorbě trojrozměrné matrice, která může v konečném důsledku pro podporu buňčné activity což je například proliferace i složité procesy, jako je diferenciaci. Nanovlákná zdánlivě napodobují fibrilární přirozené tkáňové struktury. V této práci, různé biopolymery například, které obsahují poly ( $\epsilon$ -caprolactone) (PCL), acetylovaný škrob a směsi PCL a acetylovaného škrobu. Poly ( $\epsilon$ -caprolactone) potažený škrobem byly navržen jako slibný materiál pro tkáňové inženýrství. Podstatou i hlavní cílem této práce je vytvářet trojrozměrné matrice z biopolymerů electrostatickým zvlákňování. Acetylovaný škrob ze směsi PCL a škrob by mohl podpořit životaschopnost buňek. Toto bylo prokázáno metodou MTT. Výsledky v tomto případě ukázaly, že buňky se připojily na PCL na povrchu acetylovaný škrob a ukázaly vyšší životaschopnost než scaffoldu z čistého PCL. Další typ tkáňového nosiče, acetylovaný škrob potažený PC u této vzorků se prokázalo, že buňky byly mnohem protažlejší a šíření buňek bylo podobné jako na PCL. Tak že, acetylovaný škrob ve směsi s PCL a škrobem potažené PCL by mohly být potenciálně využity jako náhrada PCL co by slibného materiálu pro tkáňové inženýrství. Výsledky testu MTT se významně nezměnily při porovnání původního syntetického polymeru, PCL. Další úsilí v budoucnosti bude zřejmě nutné pro zlepšení charakteristik nověvyvinutých materiálů.

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Beru na vědom, že si svou diplomovou práci mohu vyzvednout v Univerzitní knihovně TUL po uplynutí pěti let od obhajoby.

V Liberci

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Kind wishes,  
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## ***1 Introduction***

Production of synthetic filaments using electrostatic force has been obviously known during decades ago. Such process is the so called electrostatic spinning, or electrospinning (Subbiah *et al* 2005). In other words, electrospinning is already known as a processing technique to fabricate fibers using electrical, rather than mechanical forces from polymer solution or melt (Arumugam *et al* 2009). This technique, invented in the 1900's (Colley 1902, Morton 1902), makes use of an electrical field that is totally applied across a needle tip, from which polymer solution or melt comes out, and a collector made of generally a conductive metal, to eject a polymer jet out from a small hole. As the solution travels, the solvent gradually evaporates and leaves behind a charged fibrous mat, which is elongated by a highly electric field to the grounded collector on which fibers will be deposited. To date, many polymers either synthetic or natural have been successfully electrospun into nanofibers (Tan *et al* 2005) such as poly( $\epsilon$ -caprolactone)(PCL), starch acetate, starch-blended PCL by which this work was conducted.

The electrospinning process in this task was performed in the laboratory of Nonwovens Department, Faculty of Textile Engineering, Technical University of Liberec. The process in fact is easily to carry out, it is difficult both to control and to gain a physical understanding of the process though. Additionally, the effects of selected polymer properties on fibers features have been studied and strongly correlated to primary parameters of polymer solution for instance, viscosity, surface tension, dielectric properties of solvents, and secondary parameter to prognosticate the morphological fibrous mats (Arumugam *et al* 2009). Finally, electrostatic spinning of fibrous material opens the door to a completely new dimension of biomaterial research (Boudriot *et al.* 2006), such as exploring the application of nanofiber mats as a tissue-engineered scaffold (Ma *et al* 2005). Such technology allows for the fabrication of ultra-fine fibers with the diameters ranging from several microns to nanometers.

Starch and starch derivatives, both are in the granular and destructured form, have been blended with numerous such biodegradable polymers as polyethylene (PE), PVA, poly(ethylene-co-acrylic acid) (Koenig *et al* 1995), poly lactic acid (PLA) (Park *et al* 2000), PCL (Gomes *et al* 2008). Starch helps to improve biodegradable

characteristics to some synthetic polymers as well as sustain microbial growth in the blended form with synthetic polymers (Koenig *et al* 1995). In addition, the fibrous mats made up of starch-based synthetic biodegradable polymers (e.g. starch-blended PCL, starch-compounded PLA) have been recently fabricated using electrospinning (Gomes *et al.* 2008) and revealed promising properties envisaging their use in a wide range of biomedical application (Marques *et al.* 2005) for examples tissue engineering scaffolds, bone cement, drug delivery. The biocompatibility, degradable products, as well as non-toxic effect are some of reasons why starch is potentially preferable to be used in medical treatment (Reddy *et al* 2009).

The acetylation of starch is a chemical modification (de Graaf *et al* 1998) of numerous chemical procedures to modify naturally occurring starch. Starch acetylation can be conducted with relative ease to improve significantly the physiochemical and other functional properties of starch, even with the low level of degree of substitution (DS). The synthesis of starch acetate was chemically carried out in the laboratory of Chemistry Department, Technical University of Liberec. Furthermore, the synthesis was performed for 5 h to fabricate starch acetate with elevated DS. During the acetylation process, the free hydroxyl groups on C<sub>2</sub>, C<sub>3</sub>, and C<sub>6</sub> of the potato starch can be substituted with acetyl groups (Xu *et al* 2004). Recently, several attempts have been made to fabricate starch and other polysaccharide acetates for medical applications. Furthermore, the films of starch ester having DS in the range of 1.9 to 2.6 were made and applied in the bovine albumin serum (BSA)-released. Another report emerged, the degree of acetylation of chitosan has profoundly affected in the cell culturing process, namely cell adhesion and proliferation (Reddy *et al* 2009).

The fact that articular cartilage has little or no capacity for effective repair following traumatic pain has been recognized by clinicians for more than 250 years. To date, the natural history of joint degeneration following injury resulted is not obviously understood, though it is clear that articular cartilage often leads to symptomatic pain and ultimately to osteoarthritis (McPherson *et al* 2005). Osteoarthritis commonly sways people aged 60 and older, at about 80 % (da Silva *et al* 2008). Various surgical methods have been developed during the past 30 years to completely solve the problem of cartilage injury. The most conventional procedure to cartilage injury is debridement and lavage. A few years later, surgeons developed several techniques to restore the previous methods, such as abrasion arthroplasty,

microfracture and subchondral bone drilling (McPherson *et al* 2005). These procedures are able to improve joint function, even though the patient's mobility is probably limited, and other problem such as at the interface of bone may possibly occur. Thus, the need for improving those previous methods should be done. Moreover, the development of solution and the patient's quality of life is still probably far from ideal, although the surgical issue is initially good. Tissue engineering has recently represented an alternative route to current treatments. Its strategy may be based on (da Silva *et al* 2008) developing and fabricating therapeutic agents that utilize the combination of matrix scaffolds, that are produced via electrospinning, with viable human or animal cells, aimed to repair, restore or regenerate cells or tissue damaged caused by injury, diseases and congenital defects (Venugopal *et al* 2006). In this work, the fibrous mats were required as potential templates to accommodate chondrocytes cells.

### ***1.1 Goal and Objectives***

The goal of this work is obviously to generate three dimensional matrix scaffolds of PCL, starch acetate-compounded PCL, and probably, starch acetate-coated PCL at certain concentrations by means of electrospinning. The nonwoven fibrous mats released are intended to be appropriate templates onto which chondrocytes, the cells which are only found in cartilage, will be cultured. Another essential goal of this research is the starch based scaffolds could promote the primary step of cellular behaviors, cell viability, demonstrated by a Method of Transcriptional and Translational (MTT) assay. They might further have similar performance to PCL-based scaffold.

This study is concentrated into three main steps, conducting starch modification, carrying out electrospinning method to yield fibrous mats, thereafter culturing the chondrocytes into the resultant fibers. The specific objectives of each process are mentioned below:

#### ***Electrospinning:***

- 1) To properly design the electrospinning apparatus.
- 2) To gauge viscosity and surface tension of polymer solutions. These physical variables comprise primary influencing parameters.

- 3) To fully apply material and system parameters on the electrospinning process.
- 4) To examine solvent characters used to solubilize various types of samples, namely starch-compounded PCL, PCL, starch acetate-coated PCL.
- 5) To characterize morphological filaments by using the Scanning Electron Microscope (SEM).

***Cell cultivation:***

- 5) To culture chondrocytes cells on the surface of the constructs. One of the cellular activities of cells such as, cell viability can then be observed by an MTT assay.
- 6) To examine histological staining using confocal microscope.

***Additional point***

- 7) To provide new information regarding starch acetylation and its usage as potential scaffolds in tissue engineering, including how to synthesize the modified starch.

## ***2 Review of literature***

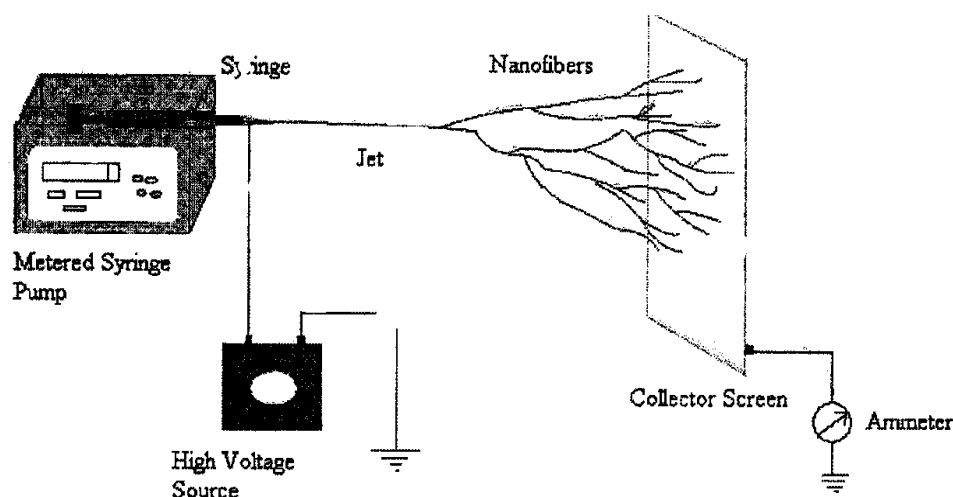
The state of art is mainly composed based on three main components reported in this work, electrospinning, the materials used such as PCL , starch acetate, as well as tissue regeneration.

### ***2.1 Electrospinning***

Electrospinning is a new method developed to create filaments. By this subchapter, all information concerning with electroprocessing will be conceptually described starting from its definition, the influencing parameters, etc.

#### ***2.1.1 Description of Electrospinning***

Electrospinning has been broadly known since 1900's (Morton, Colley 1902). This process has been recently reused for such application as high performance for filters (Doshi and Reneker 1995), and fibrous scaffolds for tissue engineering (Chew SY *et al* 2006) that utilize the characteristics of the high surface area provided by the fibrous mats (Khil *et al* 2004). Electrospinning of nanofibers thus opens the door to completely new dimension of biomaterial research (Boudriot *et al* 2006). Nanofibers have diameters from tens to hundreds of nanometers (Rakov 2006). This process utilizes a high electric force generated between a polymer solution contained in a syringe with a capillary tip and a target. Furthermore, the process can be adjusted to control fiber diameter by regulating the voltage, polymer solution concentration, and solvent composition (Khil *et al* 2004). Additionally, the electrospinning setup, in this work, is depicted in materials and experimental methods' page, subchapter 3.5 regarding polymeric matrices fabrication. Generally, the schematic of electrospinning process is shown in Fig.2.1 below.



**Figure 2.1** The schematic of electrospinning process (Courtesy: Subbiah *et al.* 2005. Electrospinning of Nanofiber)

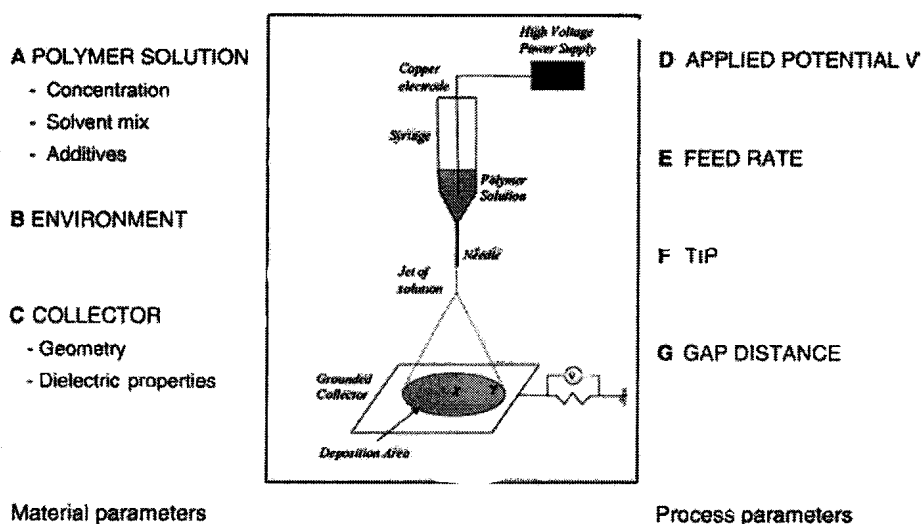
Electrospinning has been applied to technical polymers as well as biopolymers, and fibers can be spun from organic solvents, from water and also from the molten state of polymers. Accordingly, it allows to incorporate functional materials such as chromophores, enzymes, drugs, or even magnetic or semiconductor nanoparticles directly during the spinning process. The properties of the nanofibers and their arrangement in space and thus the architecture of the scaffolds can be modified in a highly controlled way by a proper selection of the spinning parameters (Boudriot *et al.* 2006). The parameters affecting nanofibrous scaffolds manufacturing may be broadly classified into material parameters and processing conditions which include the applied voltage, temperature and effect of the collector, and ambient conditions. Thus, with the understanding of these parameters, it is possible to come out with setups to yield fibrous structures of various forms and arrangements (Ramakrishna *et al.* 2005).

## 2.2 Factors Affecting Quality of Fibers

Two classes of parameters that influence electrospinning process can be identified from the literature: the first will be associated to the materials variables relating to polymer and solvent characteristics, afterward the process parameters. For convenience of these descriptions are categorized as the materials (A, B, C) and the process variables (D, E, F and G), as seen in Fig. 2.2. However, because these are

interrelated, a small change in any of these parameters can significantly impact the others, often dramatically affecting nanofibers quality or even halting the spinning process altogether (Andrady 2008).

Studies carried out by Deitzel *et al.* 2001 and Tan SH *et al.* 2005, have attempted a direct comparison of the relative importance of different variables on fiber quality. Tan and his co-workers further studied a copolymer of L-lactide-*co*-caprolactone (30% caprolactone) [P(L-CL)] in several solvents, concluded polymer concentration, average molecular weight, and the electrical conductivity of the solvent to be the dominant parameters that control nanofiber morphology. Mitchell and colleagues studied melt electrospinning of thermoplastic polyurethane (PU) using a dielectric material as an auxiliary collector that was not grounded within the gap to collect the nanofiber mats samples.



**Figure 2.2** The basic materials and process parameters involved in electrospinning of polymer nanofibers. (Courtesy: Hsu and Shivkumar 2004 )

### 2.2.1 Material Parameters

Primary or material parameters are the most affecting parameter in the electrospinning process. These parameters consist of viscosity, surface tension, dielectric properties of various solvents. Materials parameters are shortly informed as follows:

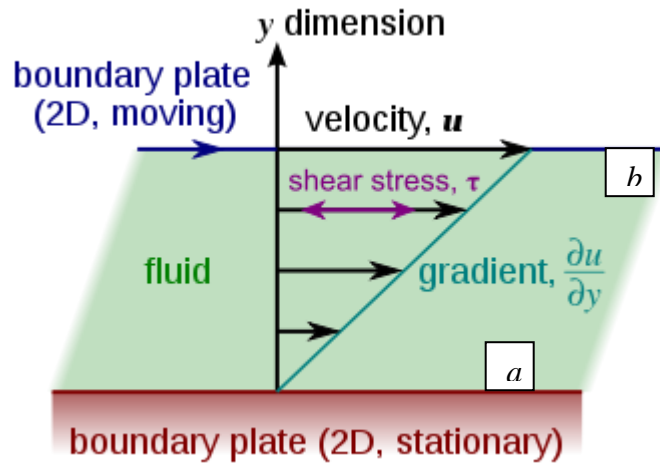
### ***Viscosity of polymer solution and concentration***

Generally, when a polymer consisting of high molecular weight is solubilized in a solvent, its viscosity will be more elevated than solution of the same polymer comprising low molecular weight. Viscosity is further one of the necessary factors to prevent the electrically driven jet when a high electric field stretches out polymer solution generating a continuous solution jet. A variable the so called chain entanglement plays critical role in this case (Ramakrishna *et al.* 2005). It obviously corresponds with viscosity of polymer solution. Additionally, the dimensionless product of the intrinsic viscosity and the concentration,  $[\eta]_c$  is potentially referred to as the Berry number. The significance of the Berry number arises from the fact that for a solution to have chain entanglements,  $Be > 1$  (Gupta *et al.* 2005). Therefore, an increased in the concentration, which similarly results in increasing the molecular weight, will result greater polymer chain entanglements within the solution which is necessary to retain the continuity of the jet during electroprocessing.

The term viscosity based on physical viewpoint refers to the description of fluid flow to characterize the degree of internal friction occurred in a fluid. The degree of internal friction is further associated with the resistance of two adjacent layers of fluid to relatively move to each other (Halliday *et al.* 2008). To gain in fact an elementary definition, we can simply use illustration as follows: consider two infinite flat plates,  $a$  at rest and  $b$  moving at constant velocity and the space between those plates being filled with the fluid under consideration. According to Newton's law of fluid friction, the velocity distribution is linear with a constant gradient,  $du/dy$  as seen in Fig. 2.3. It ultimately exhibit that the shearing stress at either wall is proportional to the velocity gradient as shown in mathematical expression below:

$$\tau_0 = \mu \frac{du}{dy} \quad (2.1)$$

The coefficient  $\mu$  is known as the viscosity, or more precisely, as the dynamic or absolute viscosity of the fluid (Kestin J and Di Pippo 1972). Such principal viewpoint is ultimately used to elucidate the measuring viscosity carried out employing RotoVisco1 as described in Chapter 3, subchapter 3.3. Prior to electroprocessing, viscosity therefore necessitates to be gauged from various polymer solutions.

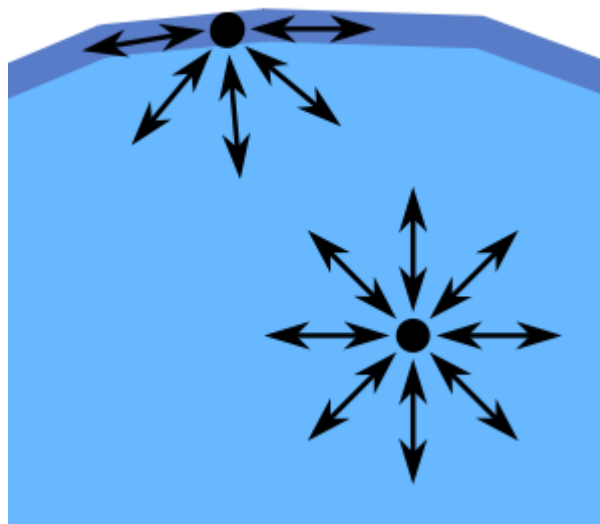


**Figure 2.3** Illustration of Newton's Law of fluid friction (copied from [http://en.wikipedia.org/wiki/File:Laminar\\_shear.svg](http://en.wikipedia.org/wiki/File:Laminar_shear.svg))

### *Surface tension*

Surface tension has profoundly impact in the electrospinning process as well. It reduces the surface area per unit mass of a fluid. In this case, once there is a high concentration of free solvent molecules, those molecules then have tendency to congregate due to the influence of surface tension. In addition, the solvent molecules will probably tend to spread over the charged solution, decreasing the tendency of solvent molecules itself to interact intensively under the effect of surface tension. Such condition will possibly occur whether the polymer solution consisting of high molecular weight (Ramakrishna *et al.* 2005).

According to physical standpoint, surface tension is caused by the attraction occurred between the liquid's molecules by various intermolecular forces. In the bulk of liquid, one molecule is equally attracted by other neighboring molecules. Such condition is truly distinct with molecule arrays placed on the top layer of liquid which are strongly pulled inwards deeper by other liquid molecules and are not attracted by the neighboring molecules as seen in the bulk of liquid. Such phenomenon is illustrated in Fig. 2.4 below.



**Figure 2.4** Illustration of the liquid molecules attraction (The image taken from <http://en.wikipedia.org/wiki/File:Wassermolek%C3%BCleInTr%C3%B6pfchen.svg>)

The one of several formulas involving surface tension is the expression for capillary force, given by

$$F_c = \gamma \cos \theta \quad (2.2)$$

where  $\theta$  elucidates the contact angle between the vector, representing the surface tension and the plain of perimeter (Lukas *et al.* 2009).

Surface tension alongside viscosity of polymer solution is very important parameters in the electrospinning process. Both of which strongly enable to affect the fiber formation and the structural morphology of electrospun fibers. The same case as viscosity, surface tension is necessarily to be measured using tensiometer, Kruss-K9, which are deeply illuminated in subchapter 3.3, precisely 3.3.2.

### ***Dielectric properties of solvents used***

The dielectric constant  $\epsilon$  of material is essentially the ratio of permittivity of a substance to the permittivity of free space. Hence, it does express the extent of a material involved to concentrate in the presence of electric flux ([http://searchcio-midmarket.techtarget.com/sDefinition/0,,sid183\\_gci548179,00.html](http://searchcio-midmarket.techtarget.com/sDefinition/0,,sid183_gci548179,00.html)). Solvents with different values of  $\epsilon$  in electrospinning will interact very differently in the electrostatic field. With solutions of high dielectric constant, the surface charge density on the jet

tends to be more evenly dispersed. This translates into high nanofiber quality and productivity during the electrospinning process (Andrady 2008). Generally, a solution containing high dielectric constant possibly reduces the beads formation and the diameter of the resultant electrospun fiber (Ramakrishna *et al.* 2005). In addition, this research strongly utilized organic solvents such as chloroform/methanol-mixed solvents, chloroform/DMF in a certain ratio. Those solvents can be employed to solubilize PCL with which mixed-solvents take a part in the jet forming process. The whole of preparation methods of various polymers to set up in the form of solution are highlighted clearly in Chapter 3, subchapter 3.2.

### ***2.2.2 Process Parameters***

Another important parameter in electroprocessing is process parameters which include high electric field, flow rate, collector, nozzle to collector distance (NCD), and the nozzle tip.

#### ***Electric field strength***

Various instability modes that occur during the formation of filaments are expected to occur by the combined effect of both the electrostatic and the material properties of the polymer solutions. In electrospinning, the charge transport caused by the applied voltage is mainly due to the flow of polymer jet towards the collector, and the increase or decrease in the current is attributed to the mass flow of the polymer from the tip of capillary (Subbiah *et al.* 2005). Depending on the flow rate of the solution, a higher voltage may be required in order to get a stable Taylor Cone. The columbic repulsive force exhibited in the jet will then stretch the viscoelastic force. In addition, if the applied voltage is higher, the greater amount of charges will cause a highly charged jet to accelerate faster and then more volume of polymer solution will be ejected from the nozzle tip. The Taylor Cone may recede into the capillary tip whether the flowing of the polymer solution from a needle travels faster towards the collector than the supply from the source.

### ***Flow rate***

The feed rate will obviously determine the amount of solution needed in the electrospinning process (Ramakrishna *et al.* 2005). Moreover, the flow rate of the polymer solution is an important process parameter since it influences the jet velocity and the material transfer rate. Some recent papers have demonstrated that the fiber diameter and the pore diameter increased with an increase in the polymer flow rate (Subbiah *et al.* 2005).

### ***Collector***

Generally, collector in electroprocessing is made out of conductive material such as aluminium foil which is electrically grounded in order to get a stable potential gap between the source of jet and the collecting device (Ramakrishna *et al.* 2005). Contact occurred between charged nanofibers and the grounded collector surface generates the removal of electrical charge on the fibrous mats. However, only the charge on the first layer of nanofibers will be able to release out rapidly. The discharge in subsequent layers will be slower because polymeric matrices are good electrical insulator (Andrady 2008).

### ***Nozzle-collector distance***

In some literature, the traveling time as well as the electric field strength will profoundly sway electroprocessing and the resultant filaments. Besides, changing the nozzle collector distance will have an influencing impact directly in both the flight time and the electric field strength. For independent filaments to form, the electrospinning jet must be allowed time for most of the solvents to be evaporated (Ramakrishna *et al.* 2005).

### ***The nozzle tip***

Metal needles as well as those fabricated from non-conducting materials such as glass or plastic have been widely used in electrospinning. Using a sharp, pointed electrode generally provides more efficient charging of the solution. Practical

consideration in selecting tip diameters should be taken into account due to possible interference from clogging of solvent evaporation (Andrady 2008). Additionally, a smaller internal diameter of a needle tip was found to reduce the clogging as well as the amount of beads on the electrospun fibers (Ramakrishna *et al.* 2005).

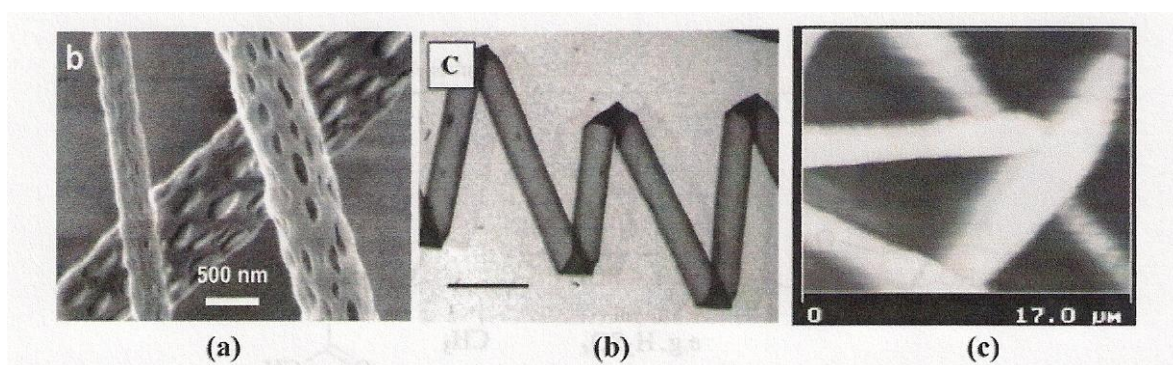
### ***2.3 Relative Importance of Variables of Electrospinning***

The electrospinning process and nanofiber mat morphology is clearly affected by a large number of variables discussed. It is challenging to attempt these variables on the basis of their relative importance in controlling specific outcomes such as very small nanofibers diameters (Andrady 2008) or a relatively large porosity (Bhattarai *et al.* 2004). Although a few common rules such as “increasing polymer solution will increase the diameter of resultant fibers” universally hold, even such generalizations must be made with caution because of the complexity of the electrospinning process. Any trends may not only be specific to a given polymer-solvent combination, but they may also be influenced by the specific set of process variables in operation. For example, as previously mentioned earlier, increasing the applied high voltage (Andrady 2008) will necessarily induce charges on the solution and together with the external electric field, will then initiate the electrospinning process when the electrostatic force in the solution overcomes the surface tension of the solution (Ramakrishna 2005). Then, by the time a high voltage is applied on the tip of syringe, it may have no effect, increase the fiber diameter, or decrease fiber diameter, depending on the process regime in which electrospinning is carried out (Andrady 2008). Another example, when poly (L-lactic acid) (PLLA) dissolved in N,N-dimethylformamide (DMF)/pyridine mixed solvent, the key parameters that control fiber morphology were reported to be polymer concentration, electrical conductivity, and the average molecular weight of the polymer (Tan SH *et al.* 2005).

### ***2.4 Characterization of Electrospun fibers***

Geometric properties of electrospun fibers such as fiber diameter, diameter distribution, fiber orientation, as well as fiber morphology (e.g. cross-section shape and surface roughness) can be observed using SEM, field emission scanning electron microscope (FESEM), transmission electron microscope (TEM), and atomic force

microscopy (AFM). The use of TEM does not require dry fibers as done on SEM. Thus, electrospun fibers from a polymer solution can be directly observed under TEM. Furthermore, an accurate measurement of the diameter of fibrous material using AFM seems to require rather a precise procedure. The fibers appear larger than their actual diameters due to the geometrical tip of AFM. Fig. 2.5 exhibits fibrous mats structures observed via SEM, TEM and AFM can also be used to characterize the roughness of fibers.



**Figure 2.5** SEM of PLA nanofibers, (b) TEM of elastin-mimetic peptide fibers (bar represents 3.3 μm), (c) AFM of PU nanofibers (Courtesy: Huang ZM, et al. 2003. A review on polymer nanofibers by electrospinning and their applications in nanocomposites)

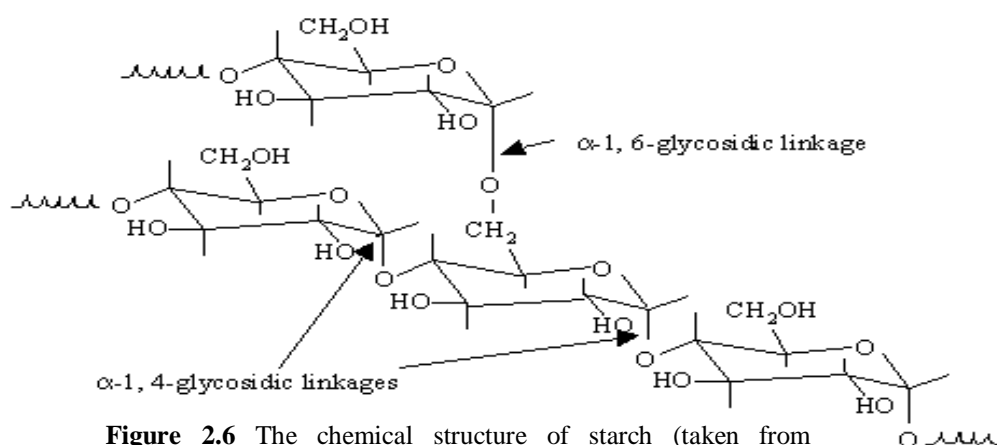
Another geometric parameter is porosity. The porosity and the pore size of nanofiber membranes are important for application of filtration, tissue template, protective clothing, and etc (Huang MZ *et al.* 2003).

The morphology of fibrous mats from this work was gauged employing another type of electron microscope. One of the advantages using this microscope is the resultant fibrous mats are not necessarily gold-coated in characterizing morphological frames. This method is highlighted more profoundly in a part of materials and experimental methods, subchapter 3.6.

## 2.5 Starch

Starch is the major form of carbohydrate storage in green plants and is considered as the second largest biomass, next to cellulose, produced on earth. Starch is a naturally occurring polymer that consists of six-member-ring glucose units (glucopyranose). In contrast to cellulose, glucose units in starch are linked by  $\alpha$ -1-4

bonds instead of the  $\beta$ -1-4 bonds found in cellulose. The  $\alpha$ -1-4 linked starch displays random-coil conformation, whereas  $\beta$ -1-4 bonds display a ridged linear structure (as depicted in Fig. 2.6). The majority of starch molecules have highly branched structures, known as amylopectin. Amylose is a primarily linear molecule that has few branches. In certain high-amylose varieties, the amylose content can be as high as 50% or 70%.



**Figure 2.6** The chemical structure of starch (taken from [www.nrel.gov/biomass/images/starch.gif](http://www.nrel.gov/biomass/images/starch.gif))

### 2.5.1 Chemical Structures of Starch

In addition to amylose and amylopectin, starch also contains lipids (up to 1%), residues of protein (ca. 0.4%), and trace amounts of phosphorus (up to 0.09%). Lipids, particularly, have great propensities to form helical complexes with starch (mainly with amylose). The amylose-lipid complex restricts the swelling of starch granules during cooking and results in paste of reduced viscosity and increased opaque appearance. An example is wheat starch. Wheat-starch paste has significantly less viscosity than other starches because of its high phospholipid content (Jane 1995).

Phosphate monoester derivatives are most commonly found in starch granules. Potato starch is known for its great phosphate monoester content. A method to determine and differentiate phosphorous of different chemical structures in starch particles by using Phosphorus-Nuclear Magnetic Resonance (P-NMR) was developed by Lim *et al.* 1994. Phosphate monoester, carrying multiple negative charges which repel one another, enhances starch gelatinization and dispersion. The charged groups also have enhanced ionic interactions with proteins and other cationic groups.

Starches with phosphate monoester derivatives, such as potato starch, yields pasted of increased clarity and viscosity.

## **2.6 Starch Ester**

Esterification reactions with carboxylic acid anhydrides under acidic catalyst are combined with chain degradation. This side reaction is used to adjust the degree of polymerization of the yields. Commercial cellulose acetates have degree of polymerization values in the range 100 to 360. The triacetate of cellulose is obtained after comparably long reaction times of 6 to 10 days at 60° C. The same protocol with starch leads to starch triacetates; the reaction time necessary for the formation of the starch triacetate can be shortened to 24 h by increasing the temperature to 100° C.

Moreover, the heating of mixtures of dry corn starch, glacial acetic acid and carboxylic acid anhydrides under pressure in small stainless steel-sealed pans yields starch esters with remarkable DS values. Starch acetates of DS 0.5-2.5 are obtained at temperatures of 160° – 180° C within 2 – 10 min, with almost complete conversion. Reaction rates enlarge with the increasing acetic acid concentration and decrease with increasing acetic anhydride amounts. The acetic acid remaining in the samples can be completely removed by vacuum stripping at 120-190° C. Longer reaction times (20-60 min) are required for the preparation of starch octenylsuccinates and dodecenylsuccinates having moderate DS values (~ 0.5 ) (Heinze T, *et al.* 2006).

### **2.6.1 Physical and Chemical properties of the Acetylated starch**

A comparison of the thermal stability of amylose esters provided the following order of esters: amylose hexadecanoate > amylose acetate > amylose propanoate > amylose phenylacetate > amylose benzoate > amylose succinate > amylose phthalate. Hexene, hexanone, methyl acetate, and allyl crotonate were identified as decomposition products of starch acetate. The degree of substitution of acetylated starch can be determined by enzymatic degradation, Infrared (IR) spectroscopy, viscosimetry (Tomasik *et al.* 2004), and acid-base titration (Xu *et al.* 2004). Several hours of heating of starch acetate with Calcium oxide (CaO) at 250-300° C produced calcium acetate and acetone.

Starch esterified with acetylsalicylic acid managed to dogs did not increase the acetylsalicylic acid level to any significant extent in the animal's blood serum. Alkaline hydrolysis of starch esters is easier than acid hydrolysis. The enthalpy of starch acetate formation was 143.5 kJ /mole, and acetylation reduced the susceptibility of the starch backbone to enzymatic hydrolysis and iodine uptake. The hydrolysis of starch and starch acetate in alkaline solutions obeys second-order kinetics.

### **2.6.2 Applications of Starch ester**

The acetylated starch is used to modify setback viscosity, gelatinization time, stability at low pH, storage characteristics, and other characteristics. The acetate of potato amylose is more flexible than cellulose acetate. Starch triacetate, amylose triacetate, and amylopectin triacetate are exhibited to be suitable for gel-chromatographic packings, as well as films, and fiber-forming material. Acetylated cereals have been used to fabricate biodegradable plastics. Starch acetate could partly replace caseinate in cheese, and also is able to remove sodium alginate in the printing of reactive dyes on textiles.

Starch esters are utilized as emulsifying agents, thickeners and sizes, binders for coal briquettes, paper coatings, and adhesives. Starch acetates having a DS of 0.21-0.50 were prepared by a method retaining the granular structure, and the products were miscible in hot water. Selective and non-selective neutral membranes from starch triacetate can be made. Starch acetate having a degree of esterification above 0.03 was blended with diluted alkali and urea to produce an adhesive.

Esters of higher fatty acids were used to produce special optical effects in photographic films. The acylation of hydrolyzed starch with acid anhydrides in pyridine provided products of interest for medical and cosmetics use, as well as application similar to those of acetylated starches.

Esters from unsaturated fatty acid chlorides are usually viscous soluble in hydrocarbons and turpentine, whose main applications are as varnishes, films, artificial threads, aqueous emulsions, and rubber-like plastics. Heating them in an inert gas yielded insoluble products formed by polymerization involving double bonds in the acyl moieties (Tomasik *et al.* 2004).

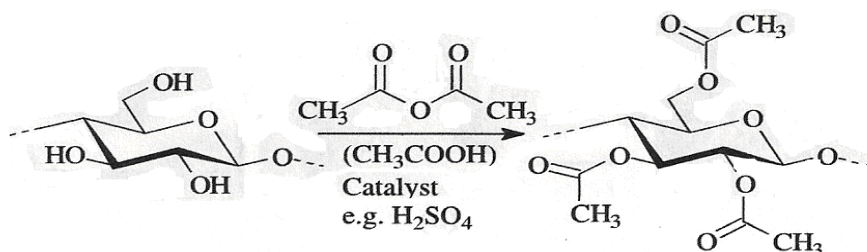
Recently, Reddy and his co-workers have influentially investigated regarding to starch acetate. They enormously exhibited that starch acetate with various DS was

able to be spun to fabricate fibers for tissue-engineered-scaffolds application. Fibers made of higher DS, for instance 2.3 and 2.8 have weight loss similar to that of PLA fibers.

### 2.6.3 Heterogenous acylation of Polysaccharides.

The most common method for the acylation of polysaccharides is the reaction with carboxylic acid anhydride in heterogeneous phase (Fig. 2.7). From the scheme exhibited in such figure, sulfuric acid acts as catalyst in this chemical synthesizing process. Acetic acid anhydride provides strongly nucleophilic ions which eventually attack hydroxyl functional group attached in the chemical structure of native starch. This work also employed catalyst, sodium hydroxide, and acetic acid anhydride to fully alter hydroxyl to ester functional group. Chapter 3 has obviously illuminated in detail of starch synthesizing process.

Cellulose acetate is the most commercially important polysaccharides ester of a carboxylic acid, and is prepared industrially or at laboratory scale by conversion of cellulose with a mixture of acetic acid and acetic anhydride in the presence of catalyst (Heinze *et al* 2006). Another one is starch acetate that can be conducted with relative ease to improve significantly the physicochemical and functional properties of the starch, even with a low DS. During acetylation, three free hydroxyl groups on C<sub>2</sub>, C<sub>3</sub>, and C<sub>6</sub> of the starch can be substituted with acetyl groups (Xu *et al.* 2004). Low acetylated starches with a DS of 0.01 – 0.2 are utilized for film forming, binding, adhesivity, thickening, stabilizing and texturing (de Graaf *et al* 1998). In contrast with that, high-DS starch acetates have been prepared since the middle of the last decades with the aim of obtaining a replacement for cellulose acetate (Xu *et al.* 2004).



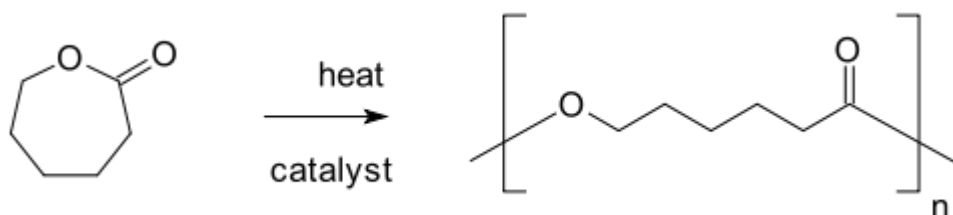
**Figure 2.7** The schematic image of conversion polysaccharide with acetic acid anhydride/acetic acid (Courtesy: Heinze T *et al.* 2006)

For better reaction control of reaction temperature and to diminish the amount of catalyst, acetylation can be carried out in methylene chloride, which is combined with

the dissolution of the products of the products formed in the final phase of reduction. Most commercial cellulose acetate is produced via this route

## 2.7 Poly( $\epsilon$ -caprolactone)

Poly( $\epsilon$ -caprolactone) , as seen in Fig. 2.8, is a semi-crystalline (Kweon *et al* 2003), aliphatic polyester that has been intensively investigated as a biomaterial (Pachence and Kohn 2005). It was miscible or partially miscible with a wide range of the other polymers, which opened the door to the use of composites as biodegradable materials (Buchanan *et al* 1995). Later, it was discovered that PCL can also be degraded by a hydrolytic mechanism under physiologic condition. Under certain circumstances, cross-linked PCL can be degraded enzymatically, leading to the process so called “enzymatic surface erosion”.



**Figure 2.8** The chemical structure of PCL (acquired from [wpcontent.answers.com/wikipedia/en/thumb/5/5f](http://wpcontent.answers.com/wikipedia/en/thumb/5/5f))

Low-molecular weight fragments of PCL are reportedly taken up by macrophages and degraded intracellularly, with a tissue reaction similar to that to the other poly (hydroxyl acids).

Poly( $\epsilon$ -caprolactone) can be synthesized by ring opening polymerization of  $\epsilon$ -caprolactones and are soluble in chlorinated and aromatic hydrocarbons, cyclohexanone, 2-nitropropane but insoluble in aliphatic hydrocarbons, diethyl ether, and alcohols (Perrin *et al.* 1997). Kweon and colleagues have investigated that a novel PCL monomer was synthesized through the reaction of PCL diol with acryloyl chloride to enhance degradability. Three-dimensional gels were formed by photopolymerization of PCL macromer, which has the potential to be used as scaffold and drug delivery matrix. The homopolymer of PCL melts at 59-64° C with a  $T_g$  of -60° C.

Another interesting property of PCL is its propensity to form compatible blends with a wide range of other polymers. In addition,  $\epsilon$ -caprolactone can be copolymerized with numerous other monomers (e.g., ethylene oxide, chloroprene, tetrahydrofuran, d-valerolactone, styrene, methyl methacrylate, vinylacetate). Toxicology of PCL has been extensively studied as part of the evaluation of Capronor. Based on a large number of tests, the monomer,  $\epsilon$ -caprolactone, and the polymer, PCL, are currently regarded as non-toxic and tissue compatible materials (Pachence and Kohn 2005).

## ***2.8 Bioresorbable and Bioerodible Materials***

Since a degradable implant does not have to be removed surgically once it is no longer needed, degradable polymers are of value in short-term of application that require only the temporary presence of a device. An additional advantage is that the use of degradable implants can circumvent some of the problems associated to the long-term safety of permanently implanted devices. A potential concern relating to the use of degradable implants is the toxicity of the implant's degradation products.

Currently four different times (biodegradation, bioerosion, bioabsorption, and bioresorption) (Khon *et al.* 2004) - which are often used misleadingly in tissue engineering literature – are of importance to discuss rationale, function as well as chemical and physical properties of polymer-based scaffolds (Hutmacher 2000) into living organisms. Generally speaking, the term of “degradation” refers to a chemical process resulting in the cleavage of covalent bonds. Hydrolysis is the most common chemical process by which polymers degrade. Moreover, degradation can occur via oxidative and enzymatic mechanisms as well. In contrast, the term “erosion” refers often to physical changes in size, shape, or mass of a device which could be the consequence of either degradation or simply dissolution. Hence, it is important to realize that erosion can be resulted in the absence of degradation, and degradation can occur in the absence of erosion as well. The terms “bioresorption” and “bioabsorption” are then utilized interchangeably and often imply that the polymer and/or its degradation products are removed by cellular activity for instance phagocytosis in a biological environment.

Today, many polymers are available which are virtually nondestructible in biological systems e.g. Kevlar, Teflon or poly (ether ether keton) (PEEK). In other words, the development of degradable polymers is relatively new area of research, but degradable materials still to be limited to cover range of diverse material properties. Thus, the design and synthesis of new, degradable biomaterials is currently an important challenge for researchers either in academic or industrial scales. The context of tissue engineering where the development of new biomaterials that can provide predetermined and controlled cellular responses is a critically needed component of most practical of tissue engineering.

Degradable material should fulfill more stringent requirement in terms of their biocompatibility than nondegradable materials. One must consider the potential toxicity of the degradation products and subsequent metabolites. The practical consequence of this consideration is that only a limited number of nontoxic, monomeric starting materials have been successfully applied to the preparation of degradable biomaterials.

**Table 2.1** Degradable polymers and representative application under investigation (Courtesy: Khon J, *et al.* 2004. Biresorbable and Bioerodible Materials)

Degradable polymer	Current major research applications
Synthetic degradable polyesters	
Poly(glycolic acid), poly(lactic acid), and copolymers	Barrier membranes, drug delivery, guided tissue regeneration (in dental applications), orthopedic applications, stents, staples, sutures, tissue engineering
Polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), and copolymers thereof	Long-term drug delivery, orthopedic applications, stents, sutures
Polycaprolactone	Long-term drug delivery, orthopedic applications, staples, stents
Polydioxanone	Fracture fixation in non-load-bearing bones, sutures, wound clip
Other synthetic degradable polymers	
Polyanhydrides	Drug delivery
Polycyanoacrylates	Adhesives, drug delivery
Poly(amino acids) and "pseudo"-Poly(amino acids)	Drug delivery, tissue engineering, orthopedic applications
Poly(ortho ester)	Drug delivery, stents
Polyposphazenes	Blood contacting devices, drug delivery, skeletal reconstruction
Poly(propylene fumarate)	Orthopedic applications
Some natural resorbable polymers	
Collagen	Artificial skin, coatings to improve cellular adhesion, drug delivery, guided tissue regeneration in dental applications, orthopedic applications, soft tissue augmentation, tissue engineering, scaffold for reconstruction of blood vessels, wound closure
Fibrinogen and fibrin	Tissue sealant
Gelatin	Capsule coating for oral drug delivery, hemorrhage arrester
Cellulose	Adhesion barrier, hemostat
Various polysaccharides such as chitosan, alginate	Drug delivery, encapsulation of cells, sutures, wound dressings
Starch and amylose	Drug delivery

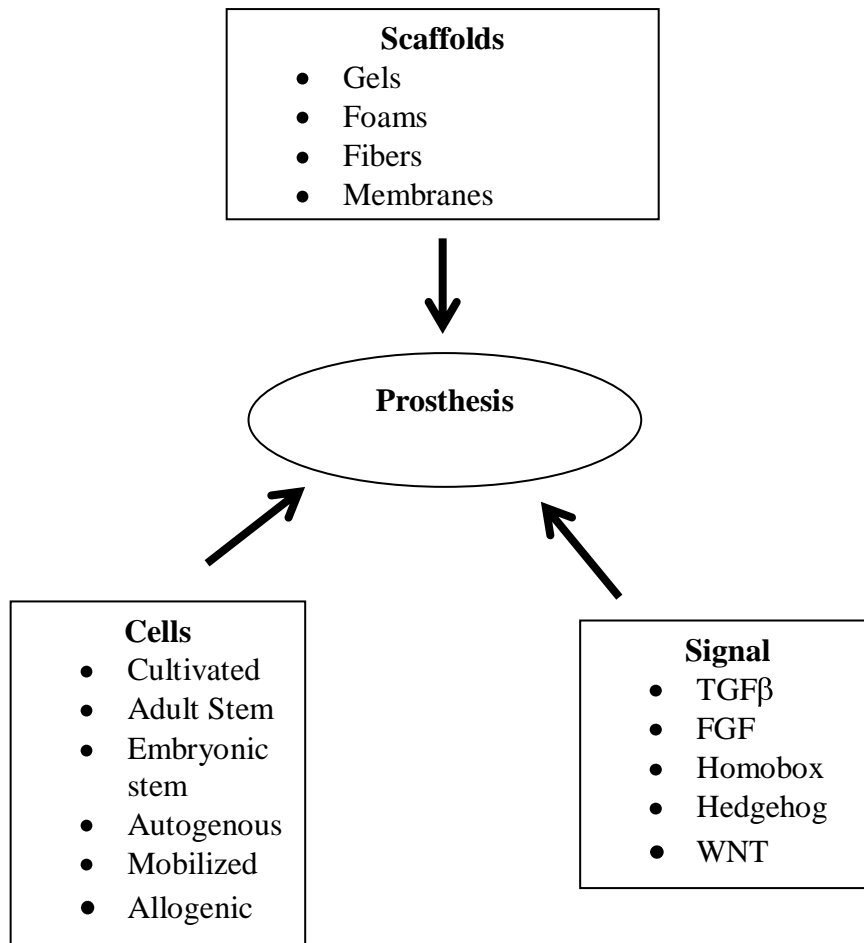
As of 1999, only five distinct synthetic polymers have been approved by Food and Drugs Administration (FDA) for use in a narrow range of clinical applications. These polymers are PLA, poly (glycolic acid) (PGA), polydioxanone, and PCL.

Recent research has led to number of well-established investigational polymers that may find practical application as degradable implants within next decades. Table 2.1 provides an overview of representative degradable polymers. It is interesting from such table that a large proportion of the currently investigated, synthetic, degradable polymers are polyesters. It remains to be seen whether some of the alternative backbone structures such as polyanhydrides, polyphosphazenes, polyamides, polyphosphonates, or polycarbonates will be able to challenge the predominant position of the polyesters in the future (Khon *et al.* 2004).

## **2.9 Tissue Engineering**

Tissue engineering combines three pillars of knowledge: biology, engineering and medical science to create artificial biological substitutes as a treatment for lost or defective native tissues or organs (Novakovic 2006). Furthermore, biologic tissues consist of the cells, the extracellular matrix (made of a complex of cell secretions, then immobilized in spaces continuous with cells), and the signaling pathways, which are built up into play through differential genes whose secreted or transcriptional products are responsible for being tissue scaffold and differentiation (Bell 2005). Scaffolds should have the same requirements needed by cells to accommodate cellular activities similar to conventional substrate in terms of low toxicity, often with the additional requirement of a three-dimensional geometry (Freshney 2006)

Figure 2.9 exhibits the triad of tissue engineering, which consists of scaffolds, prosthesis, cells and signals which interacts each other to form artificial device. The principal components of scaffolds (into which the extracellular matrix is organized in actual tissues) are collagen biopolymers, most of them in the form of fibers and fibrils. Other forms of polymer organization have been utilized for tissue-engineered scaffolds. Scaffolds can be enriched with signaling molecules, which may be embedded to them or induced into them. More importantly, this figure also shows some of the commonly occurring families of cascades of genes whose products play a critical role in morphogenesis, pattern formation, and cell differentiation, process that underlie histogenesis and organogenesis. The focus of such triad is prosthesis (Bell 2005).



**Figure 2.9** The triad of tissue engineering (Courtesy: Principal of Tissue Engineering Book, Second Edition, Academic Press)

### ***2.9.1 Cartilage Tissue Engineering***

It has been postulated that articular cartilage has limited capacity for repair due to a limited supply of cells in the vicinity of the wound to mediate process of regeneration of cells. Unlike skin, for instance, in which both vasculature and adjacent tissues provide cells for mediating the wound healing process, articular cartilage contains no vascular supply. In other case, for example articular chondrocytes which, are normally involved in articular cartilage synthesis and its maintenance, are enclosed in a dense extracellular matrix that restricts their mobility and their ability to contribute to the wound healing process. Synovial cells are present in synovial fluid, but apparently their amounts are too few or their biological properties are too limited to mediate adequate repair of any but the most minute cartilage defects (McPherson *et al.* 2005).

Articular cartilages are important in load bearing and reducing friction of the articular surfaces. Due to the limited capacity of articular cartilage to regenerate itself, and cartilage defect generating from aging, joint injury cause joint pain and loss of mobility. Numerous attempts have been developed to repair articular defect of cartilages, which include transplantations of various cells or tissues, for example utilized an autologous cultured chondrocyte or autologous bone marrow mesenchymal cells transplantation in constructing articular cartilage injuries. Because cartilage is subjected to mechanical stimulus in its natural environment, a strategy to regenerate cartilage under the action of similar stimulus is typically followed, for instances, in vitro of culturing cell, in which chondrocytes proliferation can be affected, bioreactors, and biomaterial morphology can also significantly influence the quality of tissue-engineered cartilage. Tissue engineering has improved one of various ways to provide cell based therapy, particularly to repair the defect and restore joint function. Furthermore, to solve such problems above, numerous techniques have been launched to produce nanostructured biodegradable components such as microspheres, foams and films. It has been demonstrated that the molecular structure and morphology of PLA, PGA, and PCL and their copolymers play an important role in the degradation and mechanical properties of the resultant products (Ramakrishna *et al.* 2005).

### ***2.10 Chondrocyte Structure and Function***

Chondrocytes are the constituent cells of cartilage. Due to their ability in endochondral ossification, they dramatically play an important role in determining the rate of bone growth (Hunziker 1994). A chondrocyte always originates from a mesenchymal cell, the prechondrogenic cell, or chondrocyte precursor cell which is only defined by the expectation that its daughter cell will be a differentiated chondrocyte. Chondrocyte precursor cells are of vague fibroblastic appearance and synthesis – like fibroblasts – type I and type III collagen, fibronectin, and non-cartilage-type proteoglycans. Mature chondrocytes occurred can be identified on the basis of common morphological features such as spherical cell shape with scalloped edges, an extensive rough endoplasmic reticulum and Golgi apparatus, and a plentiful of extracellular matrix which stains metachromatically with toluidine blue.

At the ultrastructural level the chondrocytes (e.g. hyaline cartilage) are enclosed by a network of unbanded, 20 and 70 nm collagen fibrils, in which

proteoglycan granules are embedded. According to attempts conducted in vivo, a shift in collagen expression may be observed throughout the process of chondrocyte development. Whereas chondrocyte precursor cells primarily express type III collagen, maturing chondrocytes shift to an expression of type II and type X collagen. Otherwise, chondrocytes in the calcified cartilage region often contain thick, cross-banded collagen fibrils reminiscent of type I collagen fibrils (Meyer and Wiesmann 2006).

### ***2.10.1 Cartilage Repair with Chondrocytes***

Cell division in hyaline cartilage can be found in the growing rat and rabbit with long term labeling it is feasible to identify a population of label-retaining cells found in hyaline cartilage, perichondrium and the growth plate (Ohlsson 1992).

The aim for a cartilage repair treatment should be the induction of biological healing and regeneration of cartilage, a target that could be reached either by enhancement of the intrinsic repair potential of articular cartilage or by the introduction of cells or tissues with a potential of regenerating new cartilage.

Increasing the intrinsic repair has been conventionally concentrated on the recruitment of pluripotent cells from bone marrow by penetration of subchondral bone by drilling or manufacturing. However, the treatments usually result in fibrocartilaginous repair tissue lacking the biomechanical properties of the hyaline cartilage and with a poor long term clinical outcome (>4 years). Periosteal/perichondral transplantations combined with opening of the bone marrow has generated in the hyaline-like tissue form fabricated by the transplant or pluripotent stem cells from the bone marrow. The clinical long term yielded from these treatments are promising in a well selected group of patients with small cartilage injuries but for the majority of patients with cartilage defects the long term issue is poor mainly due to calcification of the transplants.

An alternative treatment method utilized would be transplantation of cultivated chondrocytes, since the cause of the inadequate cellular response of hyaline cartilage to injury could be due to the entrapment of the chondrocytes in the cartilaginous matrix resulting in a limited number of cells capable of a proper cartilage tissue repair. Natural and synthetic scaffolds created have also been used for repair of cartilage

defects but our own results with fibrine glue and carbon fiber matrices as scaffolds for chondrocyte implantation has been discouraging.

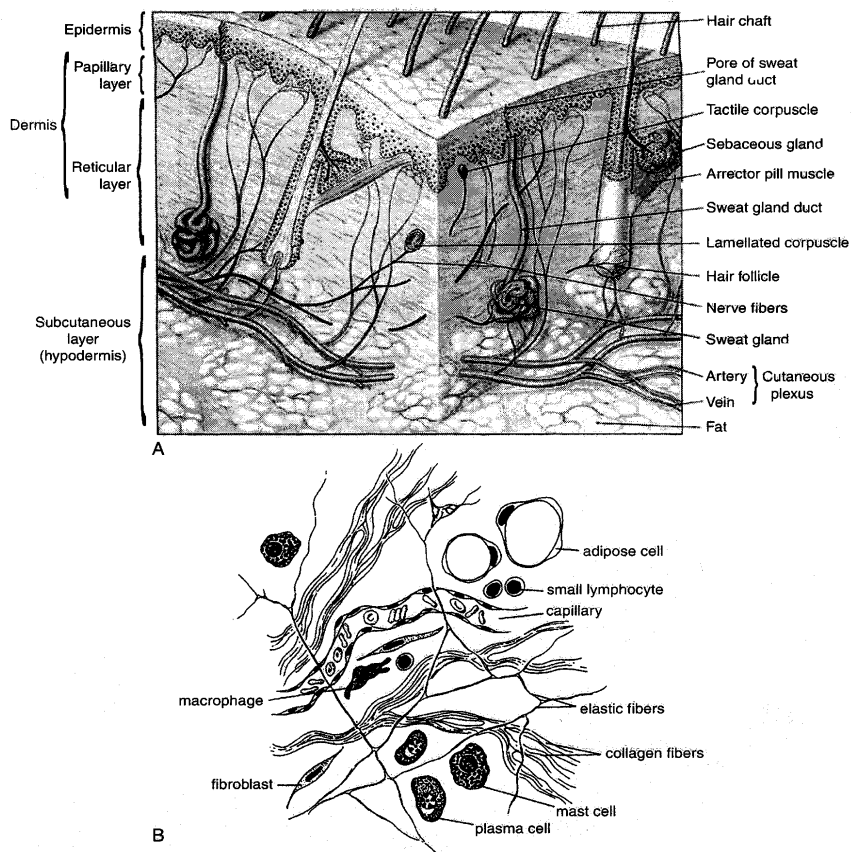
Cell therapy for treatments of tissue injuries in humans has been in limited clinical use over the last two decades (as shown in Fig. 2.10) and for cartilage the first clinical treatment attempt was made in Sweden almost 15 years ago.

- Cell therapies in clinical use
  - In Vitro fertilization (1978)
  - Cultured keratinocytes used for treatment of severe burns (1984)
  - Chondrocyte cell transplantation (1987)
  - Stem cell therapy for chronic myeloid leukemia (1988)
- Cell therapies in experimental use
  - Fetal nerve cell therapy in Parkinson's disease (1982); Xeno (1999)
  - Cultured fetal  $\beta$  cells from pancreas in diabetes
  - Corneal stem cells (2001)

**Figure 2.10** Current human cellular therapies in clinical and experimental use (This scheme is rewritten from Anders Lindahl's *et al.* article: Cartilage repair with chondrocytes)

## ***2.11 Tissues, Extracellular Matrix and Cell-Biomaterial interactions***

Biologic tissue is composed of three basic components: cells, intercellular substances, and numerous body fluids. Cells, the living component of the body, are surrounded by and obtain their nutrients and oxygen, including the body fluids blood, tissue fluid (known as extracellular fluid), and lymph. Blood consists of blood cells suspended in a slightly viscous fluid called plasma. More tissue fluid is produced than can be absorbed back into the capillaries; the excess is carried away as lymph by a series of vessel called lymphatics, which ultimately empty the lymph into blood stream (Schoen *et al* 2005).



**Figure 2.11** Organization of tissue at low and high power. (A) The components of the integumentary system (skin) demonstrating relationships among the major components. The entire diagram represents approximately 1 mm. (B) Diagrammatic representation of cells and fibers of loose connective tissue. The entire diagram represents approximately 100  $\mu$ m. The Scheme A is taken from Martini, F.H., 2001. *Fundamentals of Anatomy and Physiology*. Prentice Hall, Upper Saddle River, NJ. B, is taken from Cormack, D.H., 1987. *Ham's Histology*, 9th. Lippincott, Philadelphia.)

Extracellular Matrix (ECM) comprises the biological material yielded by, residing in between, and supporting cells. Extracellular matrix, cells and capillaries are physically integrated in functional tissues (as displayed in figure 2.11). Extracellular matrix holds cells together by providing physical support and a matrix, to which cells can adhere, signal each other and interact. During normal development and as component of the response of tissues to injury, adhesive interactions coordinate interactions with cell surface receptors and subsequently, the cytoskeleton and the nucleus (Bokel and Brown 2002).

Extracellular matrix consists of large molecules synthesized by cells, distributed to the intracellular space and linked together into a structurally supportive composite. Furthermore, ECM is composed of (1) fibers (collagen and elastin) and (2)

a largely amorphous interfibrillary matrix (mainly proteoglycan, solutes, and water).

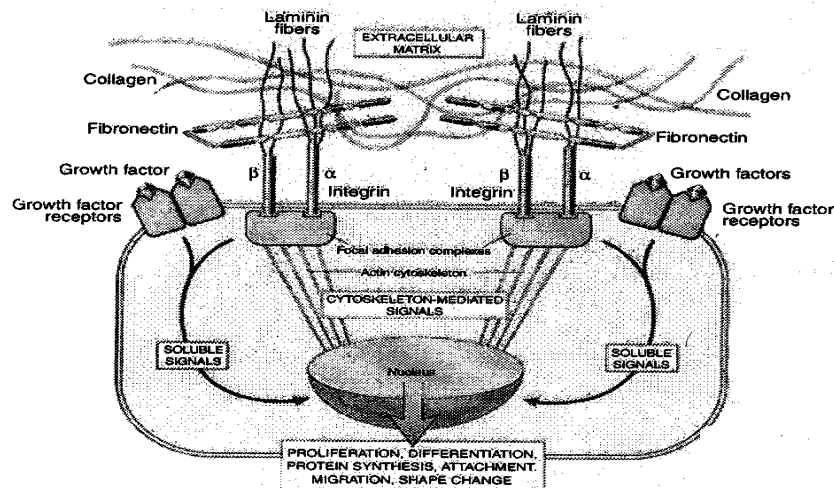
The principal functions of the ECM are:

- Mechanical support for cell anchorage.
- Determination of cell orientation.
- Control of cell growth.
- Maintenance of cell differentiation.
- Scaffolding for orderly tissue renewal.
- Establishment of tissue microenvironment.
- Sequestration, storage and presentation of soluble regulatory molecules.

Some extracellular matrices are specialized for a particular function such as, strength (tendon), filtration (the basement membranes in the kidney glomerulus), or adhesion (basement membranes supporting of most epithelia).

Matrix components and mechanical forces that cells experience dramatically influence the maintenance of cellular phenotypes and affect cell shape, polarity and differentiated function through receptors for specific ECM molecules on cell surface (for example integrins). The resultant changes in cytoskeleton and in production of the second messengers can modify gene expression. ECM also can play an important role in cytodifferentiation and organogenesis, and as a scaffold allowing orderly repair following injury. The reciprocal instructions between cell and ECM are termed dynamic reciprocity.

Like cell-cell interaction, cell-matrix interactions have high degree of specificity. It needs initial recognition, physical adhesion, electrical and chemical communication, cytoskeletal reorganization, and/or cell migration. Nevertheless, adhesion receptors may also act as transmembrane signaling molecules that transmit signaling pathways carried by transmembrane molecules from the environment outside cells to targeted molecules inside and mediate the effect of signals initiated by growth factor or compound controlling tissue differentiation (Figure 2.12).



**Figure 2.12** Integrin ECM interaction. Integrins binds ECM and interact with cytoskeleton at focal adhesion complexes (protein aggregates that include vinculin,  $\alpha$ -actinin, and talin). This can initiate the production of intracellular messengers, or can directly mediate nuclear signaling pathways. Cell surface receptors for growth factor also initiate second signals. Collectively, these are integrated by the cell to yield various responses, including changes in cell growth, locomotion and differentiation. (This image is taken from Cotran, R.S., Kumar, V., and Collins, T., 1999. Robbins Pathologic Basis of Disease, 6<sup>th</sup> ed. Saunders, Philadelphia.)

Moreover, the components of extracellular matrix, in this case ligands, with which cell interact are immobilized and not in solution. However, soluble (secreted) factors also modulate cell-cell communication in the normal and pathologic regulation of tissue growth and maturation. Cell surface adhesion molecules that communicate with ECM include the integrin adhesion receptors and the vascular selectins.

The integrins comprise a family of cell receptors with diverse specificity that binds ECM proteins, other cell surface proteins and plasma proteins, and control cell growth, differentiation, gene expression, and motility (Bokel and Brown 2002). Some integrins bind only a single component, and the other integrins can interact with several of these polypeptides (Schoen *et al* 2004).

## 2.12 Scaffold Design

Skeletal tissue, such as bone and cartilage, is usually tailored into 3-D structures in the body. For the generation and regeneration of hard and ductile tissue, such as bone, scaffolds should have high elastic modulus in order to be retained in the vicinity they were designed for, as well as, provide the tissue with adequate space for growth. If 3-D scaffold is used as temporary load-bearing device, the mechanical

properties then would maintain its load for the required time without symptoms of failure. Therefore, one of the basic problems from a scaffold design point of view is that to achieve significant strength the scaffold material must have sufficiently high interatomic and intermolecular bonding, though must have at the same time a physical and chemical structure which allows for hydrolytic attack and breakdown (Hutmacher 2000). On the other hand, scaffold investigated to date vary with respect to material chemistry (e.g., collagen, synthetic biodegradable polymers), geometry (e.g., gels, fibrous meshes, porous sponges, tubes), structure (e.g., porosity, distribution, orientation and connectivity of the pores), physical properties (e.g., compressive stiffness, elasticity, conductivity), and degradation (e.g., rate, pattern, and products) (Freshney 2006).

For tissue engineering a bone transplant, the creation of a vascularized bed ensures the survival and the function of seeded cells, which have access to vascular system for nutrition, gas exchange and elimination of by-products. The vascularization of a scaffold may be compromised by purely relying on the capillary ingrowth into the interconnecting pore network from the host tissue. In situ, the distance resulted between blood vessels and mesenchymal cells are not larger than 100  $\mu\text{m}$ . Therefore, the time frame has to be taken into account for the capillary systems to distribute through larger scaffold volume (Hutmacher 2000).

Constructs made of either synthetic or natural polymers in this work were eventually employed as templates for cell culturing, precisely articular chondrocytes from the femur chondyle of pig. Cell activities, for instance cell attachment, proliferation, as well as viability are biologically able to be perceived based on how the cells migrate over the surface of scaffolds, how the interaction yielded among cells and polymeric matrices-the cells dominantly interact each other, or the cells are able to spread over construct surface, thus providing cell-matrix interactions. The protocol to perform cell cultivation has been reported more detail in Chapter 3.

### ***3 Materials and Methods***

This chapter provides the essential steps how to carry out this work starting from the preparation of polymer solutions, the manufacturing process of fibrous scaffolds by electrospinning, and the chondrocytes culturing.

#### ***3.1 Materials***

This attempt was done by using a biodegradable synthetic polymer. This polymer was PCL. Poly ( $\epsilon$ -caprolactone) was supplied from Sigma-Aldrich with a specific weight molecular weight ( $M_w$ ) equal to 80.000 in a pellet form. Solvents were prepared to dissolve such bio-degradable polymers. Organic solvents to disperse PCL were a mixture of chloroform: DMF at 7:3 and chloroform: methanol utilized at 3:1. The organic solvents were obtained from Penta, s.r.o, Czech Republic, except methanol that was supplied from La Chema, s.r.o, Czech Republic. Both of these solvents utilized were at  $\geq 99\%$  purity. The native potato starch ( $M_w=165,000$ ) employed to compound with the synthetic biopolymers was purchased in the powder form from Lachner s.r.o, Czech Republic (Dux, personal communication).

The acetylated starch was subsequently synthesized in the laboratory of Chemistry Department, Technical University of Liberec. Such modified starch is able to dissolve in common organic solvents for instance pyridine, acetic anhydride, and formic acid. Additionally, the starch acetate that possesses degree of substitution higher than 2 is soluble in pyridine, acetic anhydride and formic acid (data not shown). If the degree of substitution is lower than 2, starch acetate probably dissolves only in formic acid. Formic acid used in this case was distributed from Lachner, sro, including acetic acid anhydride. The detailed process how to synthesize the modified starch is obviously elucidated in subchapter 3.4.

#### ***3.2 Experimental methods***

The primary protocol to perform the electrospinning process was of the preparation of polymer solutions. This work utilized a synthetic polyester group, PCL, and the well-known native biodegradable polymer, potato starch.

### ***3.2.1 Solution preparation***

Thirteen polymer solutions were prepared at certain concentrations. The preparation method was performed in a fume hood, due to organic solvents exerted for dissolving starch acetate-blended PCL, PCL, and starch acetate are somewhat hazardous solvents. Chloroform was initially added into a beaker, methanol was in turn poured into a glass jar, stirred in a few minutes prior to adding polymer scaffold, PCL. The process of dissolving PCL was completely akin once chloroform and DMF were utilized as a mixed-solvent. In order to obtain homogenous solution, all of these samples were stirred overnight by using magnetic stirrer at medium setting (about 300-400 rpm). Poly ( $\epsilon$ -caprolactone) made up in the range between 10-12 % wt. Such PCL pellets were weighed, 1 g, 1.1 g, 1.2 g, and 1.5 g for 10%, 11%, 12%, and 15% respectively. An acetylated starch, in a de-structured form, was weighed, 1.5g and 2.0g, for 15% and 20% concentration solutions, respectively. The modified starch was dissolved within formic acid, then agitated overnight. There was a sample consisted of starch-blended PCL. Such polymer solution required formic acid as a solvent. The process of making starch ester and its blended form was done by taking certain amount of starch acetate into beaker containing formic acid, agitated overnight. Poly ( $\epsilon$ -caprolactone) was then put into beaker in which formic acid-miscible starch acetate was. Total of such solution was 15% wt consisting of 1 % solution of starch acetate and 14 % solution of PCL, respectively.

### ***3.3 Testing of solution properties***

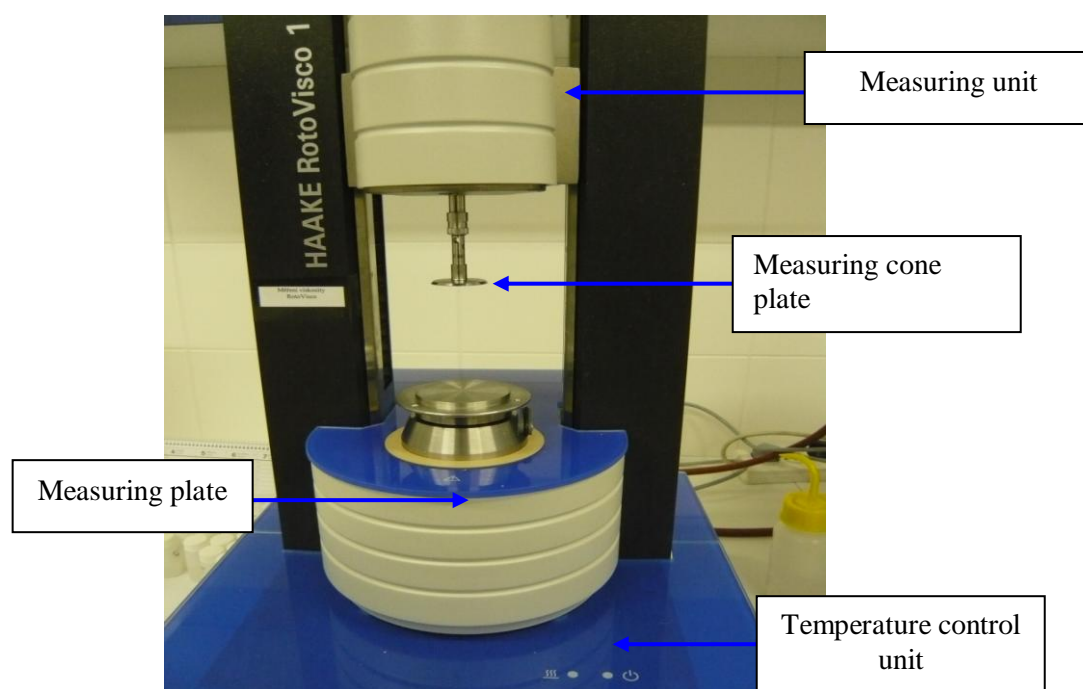
The testing of polymer solutions provides further information in accordance to physical properties that influence the spinnability of the substances investigated. The physical properties are viscosity, surface tension, etc.

#### ***3.3.1 Viscosity***

Viscosity is one of the parameters affecting electrospinning process. Viscosity may correlate to the concentration of polymers used, in turn to diameter of the resultant filaments in tissue engineering application. All of polymer samples were investigated its viscosity by using HAAKE-RotoVisco1 (Thermo Fisher Scientific,

Germany). The image of it is shown in Fig.3.1. The measuring cone plate was used due to several advantages, such as small sample volume, accurate filling, as well as easy cleaning. The description of such measuring cone is cone C35/1° H, D=35 mm (The diameter of cone is 35, and the cone angle formed among measuring plate and cone plate is 1°). The principle of this parameter is further described in subchapter 2.2.1 of cited literature.

This apparatus is facilitated by Rheo-Win-Manager software. A droplet of polymer solutions was placed on the measuring plate of RotoVisco1 by utilizing pipette. It should be addressed on the center area of the plate. The measurement was conducted at room temperature ranging between 24-25°C. The collected data of viscosity were taken and transferred into Excel Software in order to be easily calculated. The average of viscosity from data sources was then analytically determined

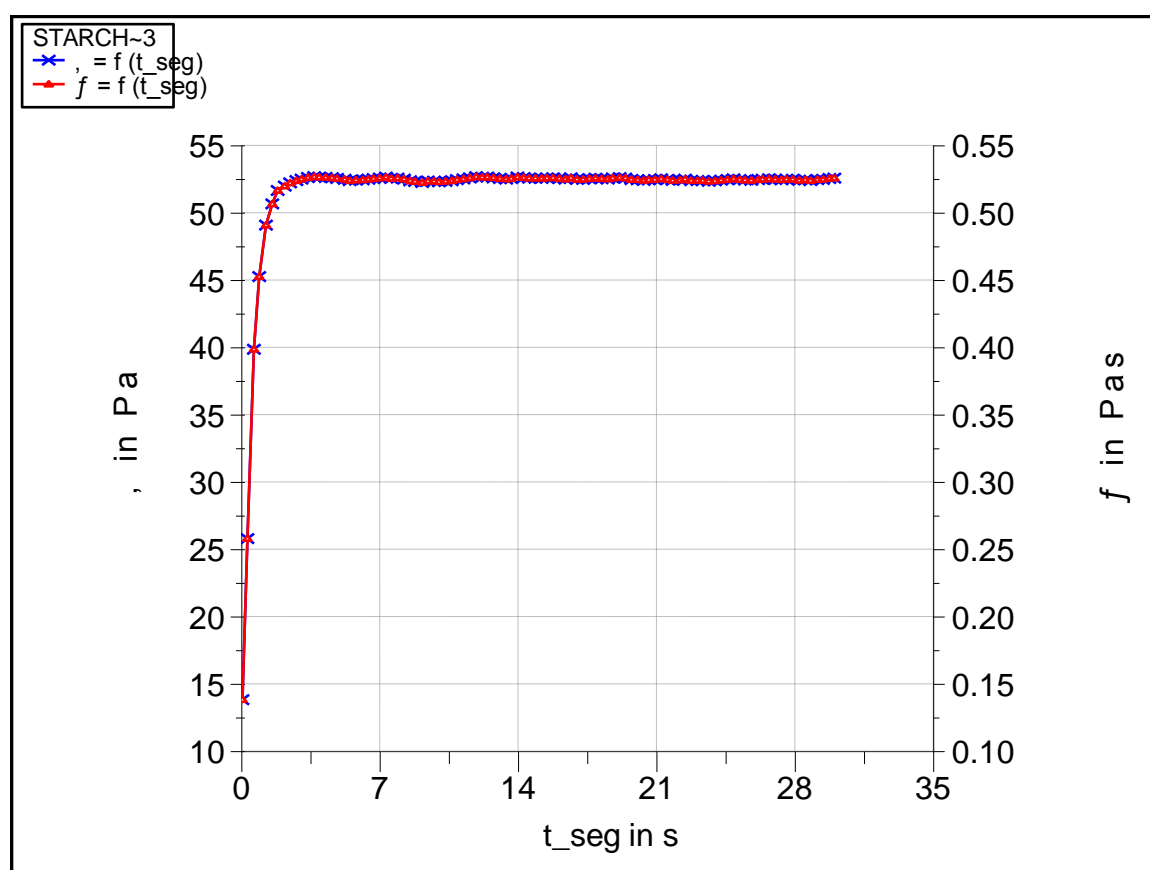


**Figure 3.1** The apparatus of RotoVisco1 to investigate viscosity of polymer solutions

The outcome after measuring viscosity is exhibited in Fig.3.2 below. There are two units parameters used, viscosity ( $\eta$ ) vs. time (t). Dynamic viscosity ( $\eta$ ) has physical unit in the pascal-second (Pa.s). One Pa.s means that one plate is moved with a shear stress of one Pascal, it then shifts towards the second plate through a distance

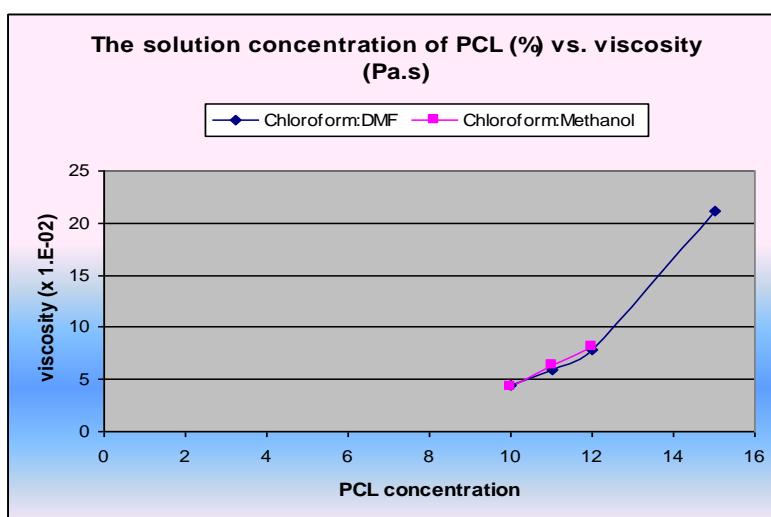
shown by the thickness of the layer between the plates in one second (<http://en.wikipedia.org/wiki/Viscosity>).

The graph given below constitutes data from an acetylated starch. As we know that the viscosity at constant shear stress and temperature may alter with time. Based on time-dependent behavior, such graph potentially shows rheotropic, or anti-thixotropic. Such behavior clearly appears that an increasing time further results in an increase viscosity. In other words, the polymer solution with increasing viscosity with time is then called rheotropic or anti-thixotropic (Chung 2000). Furthermore, the viscosity is not apparently constant in this case. From 0 to 2 s the line is linearly ascended, and then from the rest of time, 2 to approximately 30 s the viscosity resulted ranging between 50 to 52 Pascal (in logarithm). Additionally, the graph below has dominantly exhibited that the polymer solution employed can be categorized as non-Newtonian fluid. In other words, non-Newtonian fluid is a flow fluid which potentially does not result single constant value of viscosity.



**Figure 3.2** The correlation between dynamic viscosity versus time

The interrelation between viscosity and the solution concentration can be displayed in Fig. 3.3. The solution concentration has greatly impact on the viscosity resulted. An increase of solution concentration results in increasing viscosity. On the other hand, the viscosity resulted is proportionally contingent on the solution concentration as described in the Chapter 2, subchapter 2.2.1.



**Figure 3.3** The correlation between polymer concentration in two distinct solvents and the resulted viscosity

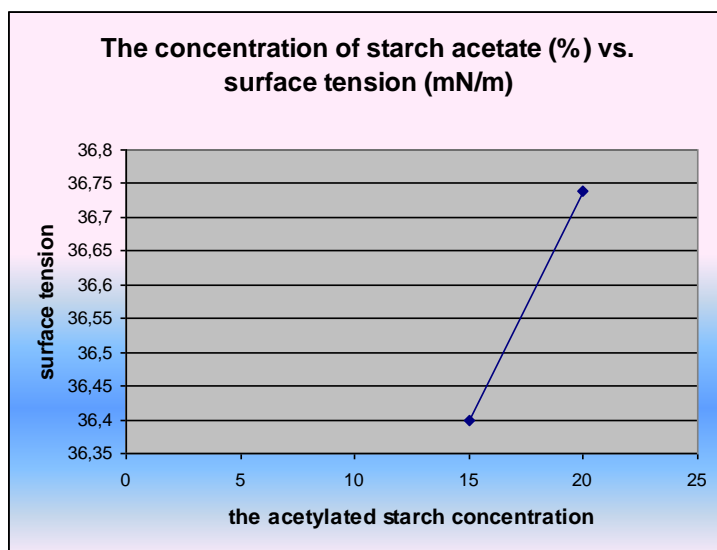
### 3.3.2 Surface Tension

Surface tension is another physical parameter in electrospinning. Surface tension should be overcome, when high applied voltage is induced at the needle tip of syringe. This principal viewpoint of surface tension itself is obviously elucidated in Chapter 2, subchapter 2.2.1. The surface tension of all polymer solutions was measured by using digital tensiometer (Kruss-K9, Germany) as shown in Fig.3.5. The method utilized to examine surface tension of each sample is plate method. The vessel for carrying out measurement should first be cleaned by acetone. It should then be cleaned under running distilled water. For the plate used (a plate method), if the plate is still not clean enough, it can be flamed by using a Bunsen burner flame. The image of plate surface utilized is clearly seen in Fig.3.6, including a vessel to place in a certain amount of polymer solutions. Each of polymer solutions taken into the vessel should be placed on the sample support. It may be necessary to lower the sample stage. Such stage should be raised by means of the coarse stage adjustment until it is just below the lower edge of the plate without wetting it, because it is helpful to

investigate the reflection of the lower edge of the plate on the liquid surface. The fine stage adjustment can be used to raise the sample stage to lower edge of the plate touches the sample surface. Mechanical drag pointer can then be set up to reference mark. The fine stage adjustment can be utilized to raise the sample stage at about 5 mm. Hence, it is certain that the plate is wetted. Such plate should be lowered until the drag pointer is pointing precisely to the reference mark. This measurement was done 5-6 times, and the resultant surface tension of each sample can then be averaged. The surface tension generated was the force pulled exerted on the plate at the breaking time of layer emerged between the plate and liquid surface.

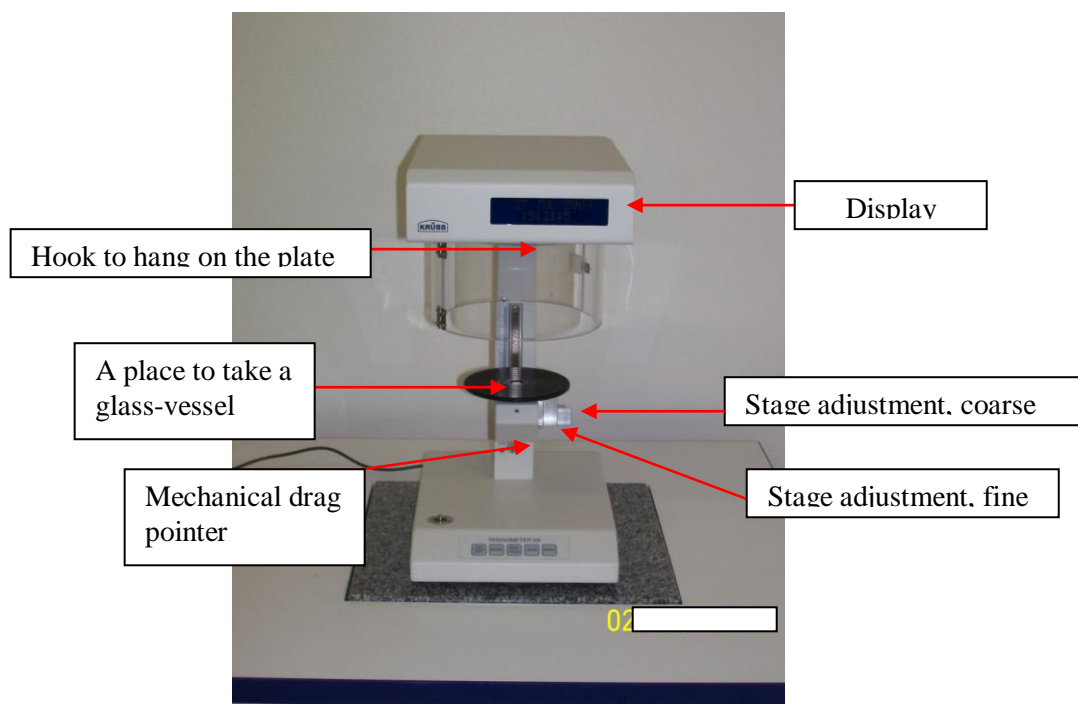
Principally, while the plate is gently directed and attached at the surface of liquid, there are potentially several forces working on it, namely surface tension, gravitational force as well as buoyant force. The equilibrium of such forces at liquid surface should be equal to zero. The net force on the object, plate, is the sum of surface tension, gravitational force minus buoyant force.

The one of example data resulted is then processed utilizing Excel as seen in Fig.3.4 below. The remainders of data yielded are able to be viewed in Appendix.

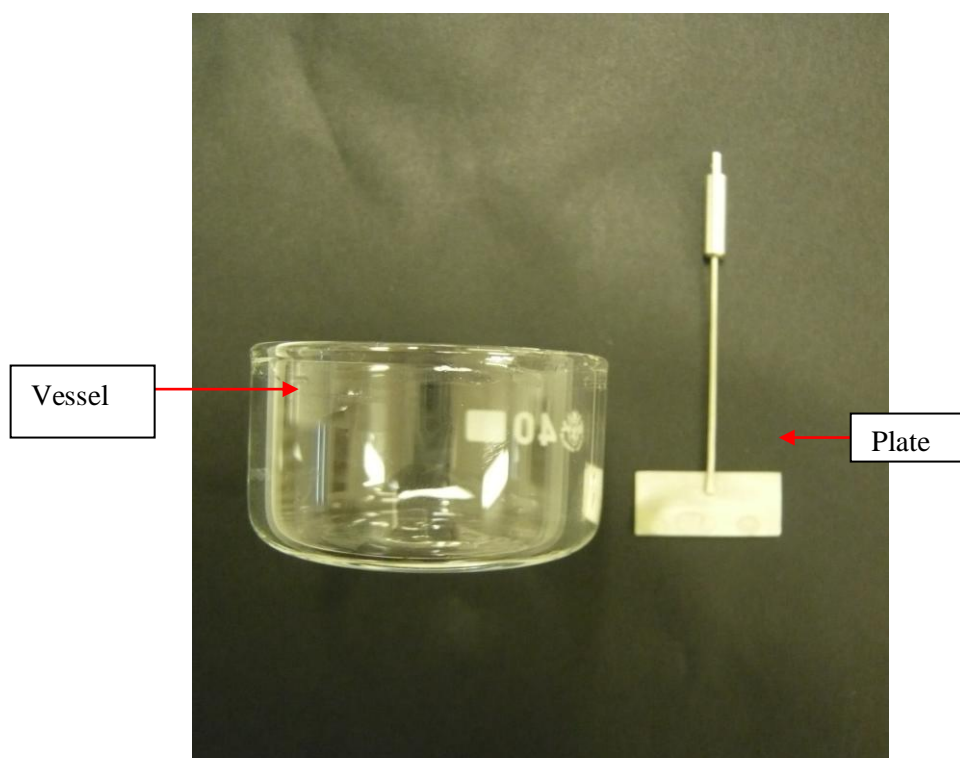


**Figure 3.4** The image of interconnection between surface tension and the concentration of the acetylated starch.

From the graph viewed above, the surface tension yielded, is strongly dependence upon the solution concentration. It linearly exhibits that the more concentrated polymer solution, the higher value of surface tension resulted. Additionally, surface tension shows a positive relationship with the acetylated starch concentration.



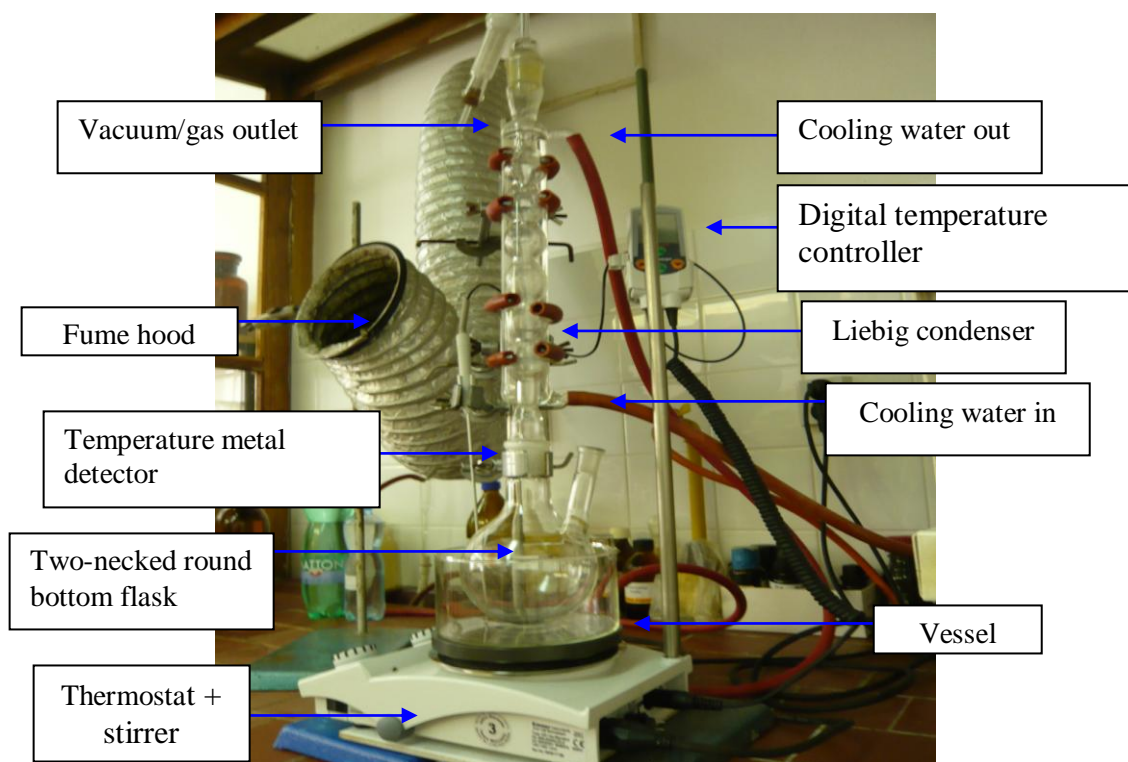
**Figure 3.5** Digital tensiometer K9 used for measuring surface tension of polymer solutions



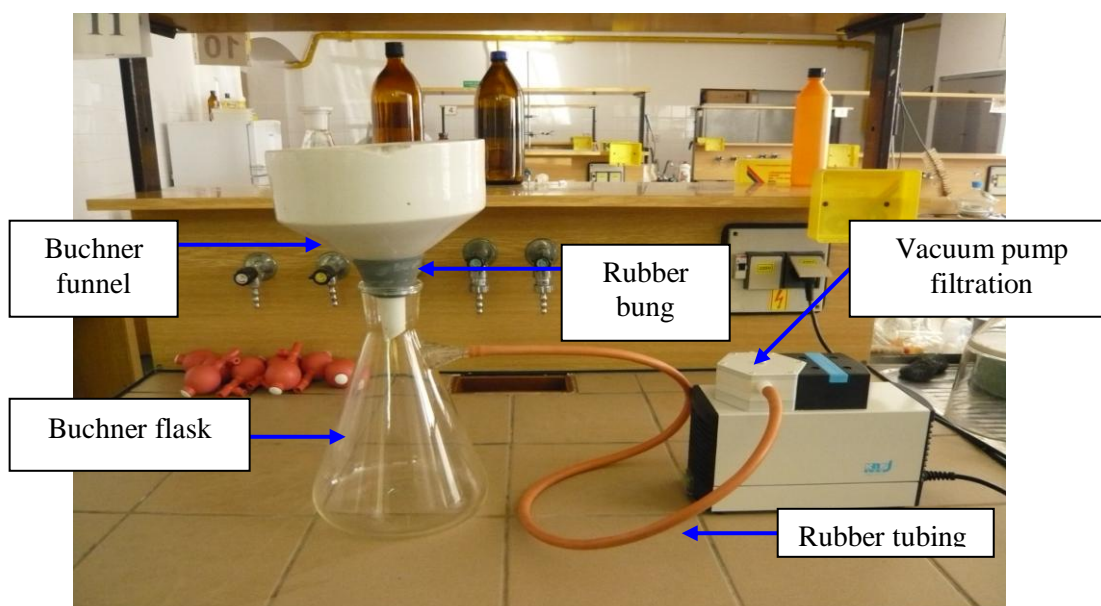
**Figure 3.6** The primary components to gauge surface tension

### ***3.4 Synthesis of Starch Ester***

Potato starch (amylose content of 15-25 % and granule size 10 – 100  $\mu\text{m}$ ) used in this research was obtained from Lachner s.r.o, Czech Republic. The measuring granule size was conducted in the laboratory of Novel Technologies and Applied Informatics, Mechatronics Faculty, Technical University of Liberec, and the result of this measurement is displayed in Appendix. The apparatus setup of the synthesizing process is depicted in Fig.3.7 below. The starch granules should be dried at approximately 50° C for 72 hours. Starch granules were weighed at about 15 gram. Dried starch was agitated with acetic anhydride. Acetic anhydride used was supplied from La Chema s.r.o, the Czech Republic. The weight of acetic anhydride performed was approximately 54 gram. Both of those substances were agitated at 250 rpm for 5 min. Sodium hydroxide was obtained from Penta sro, Czech Republic. It was used as catalyst, and was then added into the solution. The concentration of sodium hydroxide utilized was 25 % solution. The temperature at which starch synthesis carried out was maintained at approximately 123° C. The reaction was allowed to continue for 5 h under agitation of magnetic stirrer. During this synthesis, the color of solution was light-brown or a milky white. After reaction accomplishes, the mixture of substances was then cooled until 70° C by pouring amounts of tap water in the two necked round-bottom flask as fast as possible under agitation. The two necked round bottom flask was filled with tap water up to full under agitation. The magnetic stirrer was then stopped to allow acetylated starch settled. When settled, the acetic acid-consisting liquid should be decanted by lowering the manhole gradually to prevent any modified starch escaping from water. The acetylated should have a milky white color and form enormous agglomeration at the bottom of flask. The washing process utilizing tap water was done more than twice. The agglomerated starch then was removed into the conventional grinder to decline its size. The modified was furthermore transferred back into the flask under agitation. The flask then was filled with tap water, let it settled. Finally, the liquid was filtrated to get the precipitated starch ester. Figure 3.8 shows the apparatus for filtration process. This process should be carried out until the acetic acid is extracted. The final pH of the acetylated starch should be above 5 (Hanna, personal communication).



**Figure 3.7** The framework of the synthesizing process of starch ester



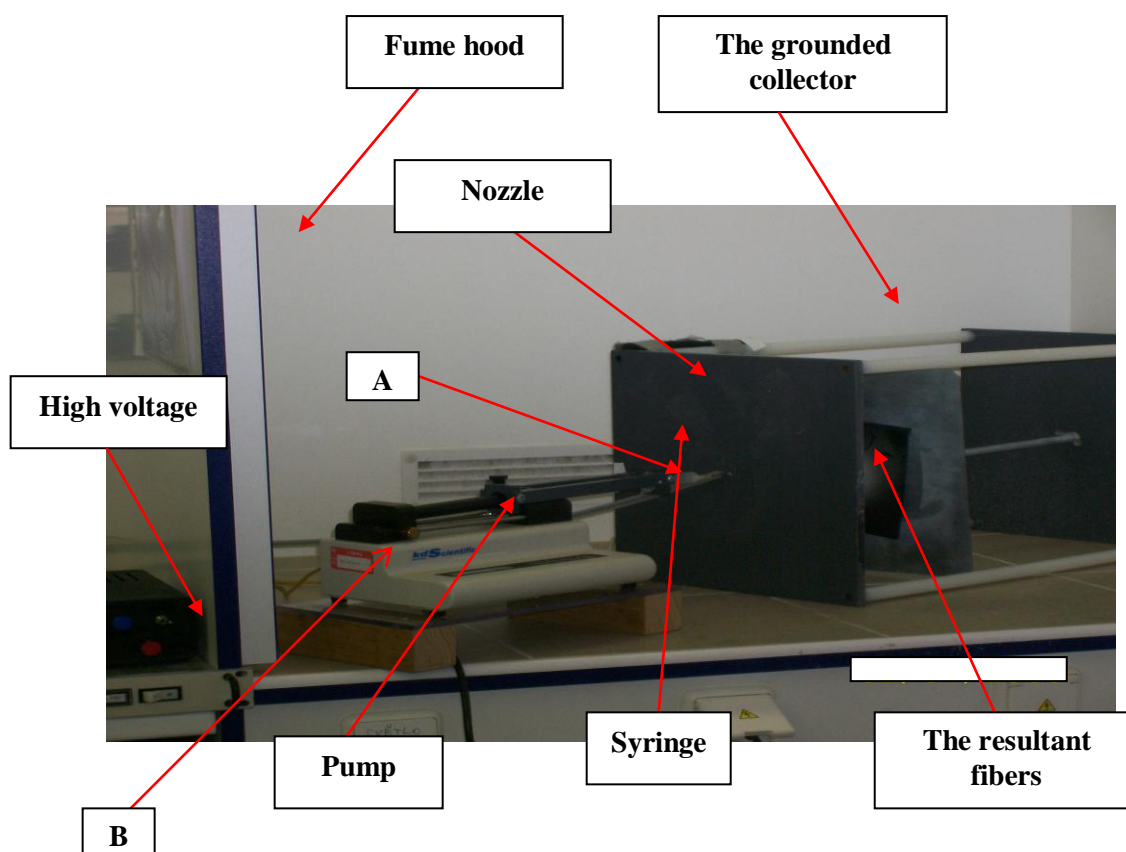
**Figure 3.8** The apparatus setup to filtrate the acetylated starch

### 3.5 Polymeric matrices fabrication

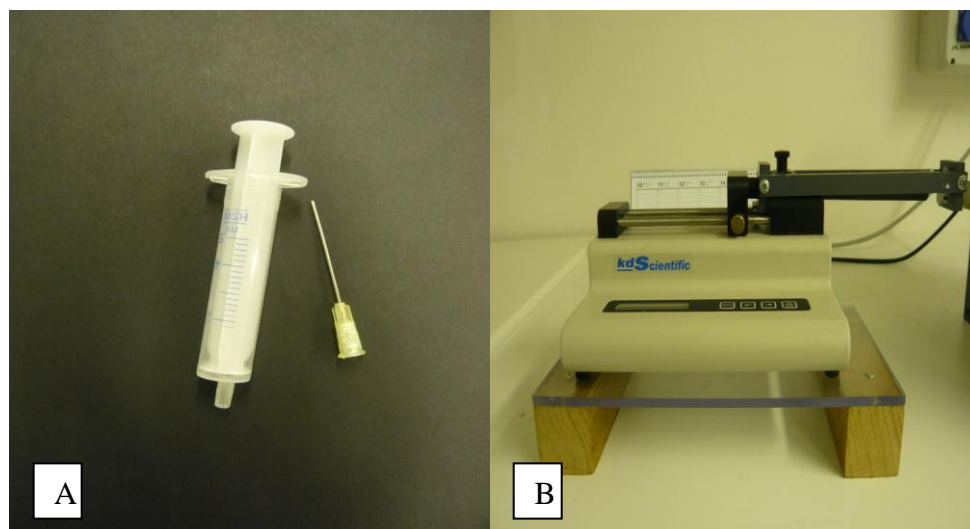
As the previously mentioned, there are two parameters the most significantly influencing the electrospinning process, that are polymer solutions and its features,

and physical. Physical parameters surely necessitate the high voltage applied, feed rate, collecting screen is usually made of the conductive metals, syringe including diameter of the spinneret, temperature, distance between the orifice and the collecting device. The electrospinning apparatus is depicted in Fig. 3.9 and 3.10 below. This attempt was conducted at room temperature, precisely at 25° C. The humidity to carry out experiment was in the range of 50-80 %. The electrostatic spinning process was completely arranged in the horizontal mode. The capillary was filled by the polymer solutions that are going to be spun. It should be linked to the syringe pump that has been set up at approximately numbers between 0.5 – 1.0 mL/h via a 20 gauge stainless-steel needle at the range of voltage between 20-40 kV. The distance between the nozzle and the grounded collector was set up ranging at 10-20 cm. A black paper or spun-bond sheet was covered on the surface of the circular-conductive metal, behaving as a collecting device. The high voltage applied and collector of electrospinning apparatus should be grounded. The power supply then was attached on the tip of the capillary. Furthermore, to make cable of power supply fixed on the needle, alligator clip can be utilized.

Two sort of biodegradable polymers utilized were, PCL, and a naturally occurring polymer, starch acetate. There was no significant case to spin PCL and a sample consisting of the acetylated starch blended PCL, because it occurred within one spinneret. Once the increased humidity was at about 70 % or higher than that, the remainders of samples comprising starch acetate and PCL were ultimately spun layer-by-layer. Polycaprolactone was finally selected as a supporting layer, means that it was initially electrospun as the first layer. It was subsequently spun to have certain thickness in order to easily be taken off, the second layer made of starch acetate was then electrospun onto the surface of polycaprolactone. The traveling time to spin starch ester was longer than getting the first layer, PCL. The total traveling time was 3 hours (an hour for PCL electrospinning and the rest was made use of coating starch ester). But, the total traveling time of starch acetate coated PCL was obviously the same as time needed to electrospin pristine PCL.



**Figure 3.9** The electrospinning apparatus

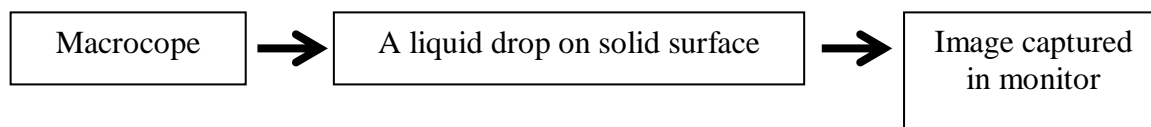


**Figure 3.10** The nozzle and syringe, B. Syringe pump

### ***3.6 Hydrophobic/hydrophilic measurement***

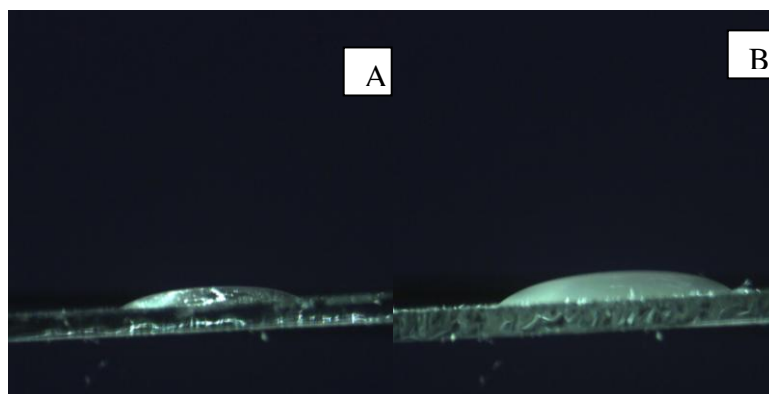
Hydrophobicity or hydrophilicity of polymer solutions is able to be measured with determining contact angle of a solution drop mounted on a certain surface. Contact angle is an angle formed when a liquid drop interacts on the surface of solid material. To determine either hydrophobic or hydrophilic of polymer solutions, some

representative samples should be prepared in liquid form. The samples were then dropped in certain volume onto the surface of glass-slide. The apparatus for taking an image of such droplets so called macrocope was set up and should be directly pointed to a droplet that will be examined. The scheme of this measurement is depicted below.



**Figure 3.11** The scheme of apparatus to investigate hydrophobicity and hydrophilicity measurement

By the assistance of certain software (Lucia G), experimenter is able to gauge contact angle of each droplet from each sample. Figure 3.12 exhibits each representative polymer solutions captured in monitor facilitated by Lucia G software.



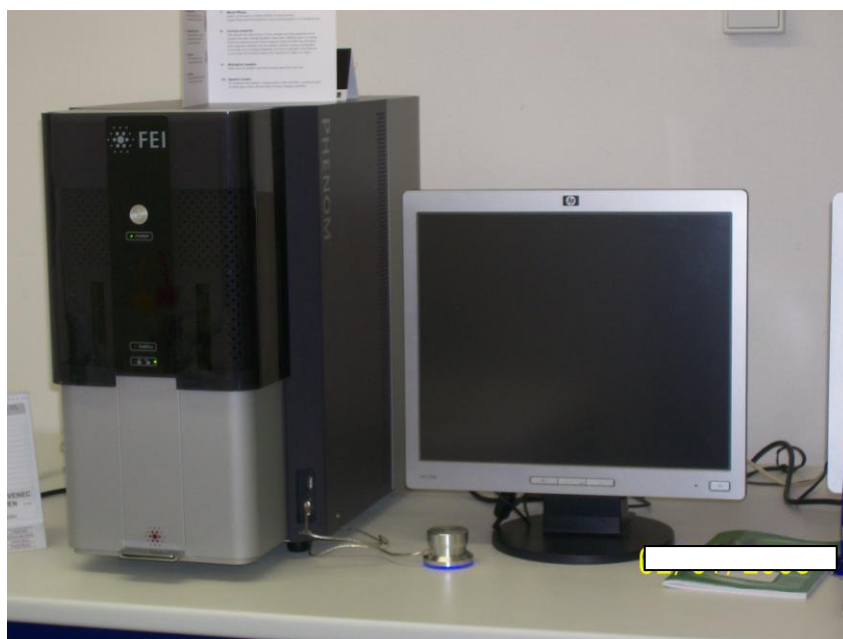
**Figure 3.12** Poly ( $\epsilon$ -caprolactone) in chloroform: DMF, B. Starch ester in formic

Generally, the contact angle of hydrophobic character is usually more than  $60^\circ$ . If one polymer is able to get more than  $150^\circ$ , such polymer can be eventually called as super-hydrophobic polymer. The average results from PCL and starch acetate after five times measurement were  $22.6^\circ$  and  $27.5^\circ$ , respectively. In fact, starch acetate and PCL should have higher contact angles than at least  $60^\circ$ , though those oddly exhibited less than  $60^\circ$ . By the time starch acetate and PCL were gently dropped onto the surface of glass-slide, those were likely to have strong attraction with the glass-slide surface. Hydrophobic polymers seemed to distribute over on which they were mounted. In other words, hydrophobic polymers, namely PCL and starch acetate seen in figures above undergo complete wetting. Wetting itself refers to the ability of the polymer solution or liquid to retain contact occurred with a solid surface. Solid

surface in this work, glass slide, does compose strong chemical bonding to link its ordering molecules. Therefore, high energy surface is needed to break its chemical bonds. In fact, there are two categorizes of energy surface, high and low energy surface. Glass containing high energy surface potentially achieve complete and partial wetting as seen in Fig. 3.12. Conclusively, the solid surface, more precisely its roughness, can influentially impact the interaction between the dropped liquid or polymer and solid surface itself, thus eventually providing the contact angle among them

### ***3.7 Testing of the morphological fibers.***

The morphology of fibers made of biodegradable polymers was tested by using the SEM, Phenom microscope. The main concept of this apparatus is that a highly charged of electron impinges on the morphological top layer of filaments that further provides signaling information about the sample surface's morphology. The measurement of morphology was carried out at room temperature at about 25° C. The humidity at room temperature was ranging among 70-80 %. The fiber samples should be cut into small-rectangular shapes, for example 1 x 1 cm<sup>2</sup> or less than such area. The samples were mounted on the microscope sample holders with double-sided adhesive tape. It is not necessarily gold-coated prior to testing all samples prepared. The fibrous mat samples containing their holders were inserted to the vacuum chamber to be investigated its morphological fibers formed. The apparatus to investigate the morphological fibers resulted can be viewed in Fig.3.13.



**Figure 3.13** The SEM

### ***3.8 Cell Cultivation***

The protocol was carried out at the Institute of Clinical and Experimental Medicine in Prague.

#### ***3.8.1 Cell culture conditions***

Chondrocytes were isolated from the femur condyle of a swine under general anesthesia (60 mg/kg ketamine and 4 mg/kg xylazine). The cartilage was cut into small pieces (1 × 1 mm). It was then put into a collagenase solution (1 mg/mL, collagenase crude 816 PSZ, Sevapharma) and incubated in a humidified incubator (37 °C, 5 % CO<sub>2</sub>) for 14 h. The cells were then centrifuged at 300 G for 5 min and seeded into culture flasks. The chondrocytes were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 % fetal bovine serum (FBS), penicillin/streptomycin (100 I.U./mL and 100 µg/mL, respectively), 400 mM L-glutamine, 100 nM dexamethasone, 40 µg/mL ascorbic acid-2-phosphate and ITS – X (10 µg/mL insulin, 5.5 mg/L transferrin, 6,7 µg/L sodium selenite, 2mg/L ethanolamine). Chondrocytes were cultivated onto the 3-D constructs at a density of 80 ×10<sup>3</sup> /cm<sup>2</sup>; the medium was altered per-three days.

Cell culture was rinsed with phosphate buffered saline (PBS). Afterwards, the fixative solution (4 % formaldehyde in PBS with 0.1 % Triton) was added and incubated for 20 min at room temperature (RT). The scaffolds were washed at approximately thrice with PBS. We ultimately added 1  $\mu\text{g/mL}$  3-Ethyl-2-[3-ethyl-2(3H)-benzoxazolylidene)-1-propenyl]benzoxazolium iodide solution [DiOC(6)] and incubated samples for 45 min at room temperature. The cultured cells onto the matrices were completely rinsed twice. Five  $\mu\text{g/mL}$  of propidium iodide was supplemented and after 10 min, they were washed at least thrice. The cell membranes and nuclei were visualized utilizing confocal microscope Zeiss LSM5 DUO (excitation at 488 nm and 561 nm and emission at 505-550 nm and 675-730 nm for DiOC(6) and propidium iodide, respectively). Propidium iodide was used as staining solution for samples C, C1, D0, D1, and D2 (the compositions of scaffolds are able to see in some figures in chapter 4, part of electrospinning).

### **3.8.2 Histological staining**

The histological staining was done to enhance contrast in the microscope image. Furthermore, it was performed on the scaffolds, namely, E0, E1, E2, F0, F1, and F2 (about scaffolds compositions are able to see in figures in chapter 4, part of electrospinning) The constructs were twice rinsed with PBS, fixed with frozen methanol for 3 min, then washed with PBS again, and finally incubated with 20 $\mu\text{g/mL}$  Alexa Fluor 488 (Invitrogen) for 1 h at RT. Subsequently, the scaffolds were rinsed three times with PBS and visualized using microscope Zeiss LSM5 DUO (excitation at 488 nm emission at 505-550 nm).

### **3.8.3 MTT assay**

The MTT test is widely used for *in vitro* measurement of the metabolic activity and proliferation of cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] is reduced to purple formazan by mitochondrial dehydrogenase in cells indicating normal metabolism. On days 1, and 7, 50  $\mu\text{l}$  of MTT (1 mg/ml in a phosphate buffered saline (PBS) pH 7.4) was supplemented to the medium (150  $\mu\text{l}$ ) and samples were further incubated at 37  $^{\circ}\text{C}$  for 4 h. Formazan crystals were solubilized with 100  $\mu\text{l}$  of 50 % *N,N*-dimethylformamide/20 % sodium dodecyl sulfate (SDS) / $\text{H}_2\text{O}$ , pH 4.7. Two hundreds micro-liter ( $\mu\text{l}$ ) of suspension was

removed to the new well and the optical density of formazan was measured ( $\lambda_{\text{sample}}$  570 nm,  $\lambda_{\text{reference}}$  690 nm). The cells cultivated onto the culture plastic, in this case, tissue culture polystyrene (TCP) were ultimately utilized as control parameter.

#### ***3.8.4 Statistics***

The quantitative data were presented as mean  $\pm$  SD (Standard Deviation). Results were evaluated statistically using One-Way Analysis of Variance (ANOVA), and the Student-Newman-Keuls Method. The level of significance was set at 0.05

## 4 Results and Discussion

There are two parts that will be distinctly outlined in this work. The first is electrospinning part, including the influencing factors. The second one will be focused on tissue engineering.

### 4.1 The electrospinning process

As previously indicated in Chapter 2, the electrospinning process is strongly swayed by two types of parameters. Material parameters such as viscosity and the solution concentration, surface tension, always initially take a part, and then followed by numerous system parameters. Consequently, it duly needs to gauge viscosity, and surface tension. The results of these material parameters (viscosity and surface tension) are obviously summarized below:

No.	Polymer solutions	Viscosity (Pa.s)	Average	No.	Polymer solutions	Viscosity (Pa.s)	Average
1.	PCL 10%- Chl:DMF	0,045193871		6.	PCL 15%- Chl:Met	0,176000000	
		0,041324516	0,043448			0,242967742	0,211086
		0,043824194				0,214290323	
2.	PCL 11%- Chl:DMF	0,059014516		7.	Starch 15%	0,382548387	
		0,069132258	0,063847			0,425483871	0,403484
		0,063392903				0,402419355	
3.	PCL 12%- Chl:DMF	0,080765161		8.	Starch 20%	0,460451613	
		0,082007419	0,081386			0,473741935	0,456151
4.	PCL 10%- Chl:Met	0,044443548				0,434258065	
		0,045011935	0,044204	9.	PCL 12%- Chl:Met	0,064378000	
		0,043157097				0,077722000	0,08621
5.	PCL 11%- Chl:Met	0,064995161	0,059099			0,094698000	

**Table 4.1** The resultant viscosity examined from each sample of polymer solutions

In this work, a variety of polymer solution groups is given, namely PCL and starch acetate. It could be seen that increasing solution concentration results in ascending of the resultant viscosity, in spite of the fact that PCL was solubilized into two distinct mixed-solvents, chloroform:methanol and chloroform:DMF. Conclusively, 12% of PCL solution enlisted the highest viscosity as compared with other various ranges in question.

No.	Polymer solutions	Surface tension (mN/m)	Average	No.	Polymer solutions	Surface tension (mN/m)	Average
<b>1.</b>	PCL 10%- Chl:DMF	29,7		<b>7.</b>	PCL 15%- Chl:Met	22,5	
		29,7				23	
		29,6	29,5			21,5	22,42
		29,5				23,6	
		29,3				21,5	
<b>2.</b>	PCL 11%- Chl:DMF	30,7		<b>8.</b>	Starch 15%	36,4	
		30,8				36,4	
		30,7	30,66			36,3	36,4
		30,8				36,4	
		30,3				36,5	
<b>3.</b>	PCL 12%- Chl:DMF	31,3		<b>9.</b>	Starch 20%	36,7	
		31,3				36,9	
		31	31,2			36,8	36,74
		31,2				36,8	
		31,2				36,4	
<b>4.</b>	PCL 10%- Chl:Met	24,1					
		23,9					
		23,2	23,28				

		22,9					
		22,3					
<b>5.</b>	PCL 11%- Chl:Met	25,9					
		24,8					
		24	24,12				
		22,8					
		23,1					
<b>6.</b>	PCL 12%- Chl:Met	25,7					
		24,5					
		23,9	24,3				
		23,9					
		23,5					

**Table 4.2** The data of surface tension given by each sample of the solution concentrations

The data exhibited above have obviously provided the same outcomes with the former physical parameter, viscosity, it means that increasing solution concentration potentially yielded ascending the surface tension yielded. Thus, the data of the solution concentrations containing PCL and starch acetate had akin track with the data given by viscosity.

Table 4.3 contributes various data of system parameters to portray the electrospinning process in question. Simply, it can be specifically categorized becoming four groups that are: 1. Fifteen percent of PCL without any starch ester, and starch-compounded PCL. Both PCL and starch-blended PCL were dissolved in different mixed solvent. The former was dispersed in chloroform:methanol at ratio 3:1, the later was then dissolved in formic acid due to the presence of starch acetate. It was not straightforward task in fact to select the appropriate solvent for starch acetate owing to its slightly chemically hydrophobic character. 2. Polymer solutions were mainly composed of PCL ranging between 10 %-12 % wt. All of those solutions are miscible in chloroform:methanol. Those PCL solutions were electrospun, they were then coated by 20 % wt solution of starch acetate dissolved in formic acid.

Group	Polymer solutions (% wt)	Voltage (kV)	Feedrate (mL/h)	Distance(cm)	Humidity
1.	PCL 15 %- Chl:Met	20	0,5	15	=<55%
	Starch-1% (PCL14%)	30	0,5	15	=<55%
2.	PCL 10%-Chl:Met	20	0,5	15	=<65%
	PCL 11%-Chl:Met	20	0,5	15	=<65%
	PCL 12%-Chl:Met	20	0,5	15	=<65%
	Starch-coated 20%	25	0,5	15	=<65%
3.	PCL 10%-Chl:DMF	20	1	15	=<80%
	PCL 11%-Chl:DMF	40	1	20	=>75%
	PCL 12%-Chl:DMF	40	1	20	=>75%
4.	PCL 10%-Chl:DMF	40	1	15	=>70%
	PCL 11%-Chl:DMF	40	1	15	=>70%
	PCL 12%-Chl:DMF	40	1	15	=<80%
	Starch-coated 15%	30	1	15	=>70%

**Table 4.3** The relation between material and system parameters, when polymer solutions are spun to produce either beads or fibers.

3. Third section is likely to have similar to the range of concentrations as beforehand. In addition, this group comprises pure-PCL which is soluble in chloroform: DMF. Those fibers or beads resulted were not coated by the acetylated starch. The last one constitutes group containing PCL (resulted from the third section, in other words, the concentrations of PCL were completely similar to those solutions listed in group three) layered with 15 % wt of starch ester.

In spite of the fact that all samples consisted of much beaded-fibers, we could still measure the average fiber diameter of each sample. The fiber diameters of C and C1 were  $431,06 \pm 29,05$  and  $236,80 \pm 15,96$ , respectively. The starch blended PCL (Sample C) presented much larger fiber diameter, probably, due to the insolubility of starch ester in an organic solvent used (da Silva *et al.* 2008) Meanwhile, the fiber diameters of D0, D1, and D2 were  $214,53 \pm 13,16$ ,  $357,01 \pm 30,10$ , and  $314,41 \pm 24,58$ , respectively. The trend of fiber diameters of samples E was similar to samples D, showing an increase of average fiber diameter from E0 to E1 with  $221,22 \pm 14,50$  and  $332,64 \pm 28,86$ , respectively. The unit of fiber diameter is in nanometer scale. The sample F0 could be measured its average fiber diameter as it almost contained no fibers. Contrary, sample F1 and F2 provided their average fiber diameters equivalent to  $230,18 \pm 11,24$  and  $230,35 \pm 14,16$ , respectively. They statistically resulted in similar fiber diameters.

All groups can be coincidentally highlighted, due to the existence of main material, PCL and starch acetate. The images of the first section can be seen in Fig.

4.1, Fig.4.2 showing D0, D1 as well as Fig. 4.3-D2 are the second section, the resultant beaded-fibers of the third group is imaged in Fig. 4.3, namely E0, Fig.4.4-E1, and Fig.4.4-E2. Finally, the last one shows visibly similar to the previous sample and pictured in Fig. 4.5 and Fig. 4.6-F2. Those figures are then compared with the extra pictures in Fig.4.6-G1 and Fig.4.7, respectively. However, PCL either solubilized in chloroform:methanol or chloroform:DMF resulted in finer fibers under normal humidity to electrospin PCL. These less beaded-fibers were generated at approximately 20-25 kV, 0.5-1 mL/h of flow rate, and at a working distance of 13-15 cm (as seen in Fig.4.6-G1 and Fig.4.7, respectively). The resultant fibers are obviously distinct, though the system parameters were set up in the same way. The aforementioned four groups displayed much more beaded-fibers rather than fine fibers shown in extra figures. The author tried to illuminate, even though the time for performing this work was not completely enough to reveal what have consequently come off in particular in the electrospinning process.

The system parameters used here, namely voltage, feed rate, and nozzle-to-collector distance, were arranged in the range of 20-30 kV, 0.5 – 1 mL/h, and 15 – 20 cm as NCD, respectively. These arrays were eventually conducted for the first and second group as jotted in Table 4.3. In general, the spectra of system parameters for the rests were nearly similar to the previous setup, adjusted in the scale of 20-40 kV, 1 mL/h, as well as 15-20 cm for the gap between the capillary tip and grounded collector, respectively. One should bear in mind here is the resultant humidity in the process of electrospinning. The humidity to spin polymer solutions was randomly in the range of 55-80%. During electroprocessing the humidity was extraordinarily high; generally the electrospinning process of PCL and starch acetate is conducted in the range of 30 – 40 %. Thus, it is not possible to achieve well-defined filaments at elevated RH (>50%). The main reason is probably the polymer solutions (PCL and PCL impregnated with starch acetate) absorb ambient water during the electrospinning process. In other words, when the polymer solutions dissolved in organic solvents are electrospun in a high humidity environment and the biodegradable polymers are hydrophobic such as PCL and starch acetate coated PCL, a bead is possibly immediately formed after the polymers interact with their environment. Thus, the absorption of water at high humidity is much more favorable than at a commonly average humidity in electroprocessing due to the higher partial pressure of water at the atmosphere (de Vreize *et al.* 2009, Medeiros *et al.* 2008).

It seems to be pretty laborious to analyze the processing parameters because the system parameters themselves were not varied in detail here, for instance the feed rate is of 0,5 mL/h for the first and second, and 1 mL/h for the fourth and fifth section, respectively. Feed rate as previously illuminated constitutes the volume that is fed out at the tip of capillary per hour. It was demonstrated less effective in affecting the resultant fibers. Other affecting factors such as high applied voltage can be clearly described here. Some studies were apparently reported that the high voltage applied was not significantly influence the electrospun fiber diameter. Applied voltage might profoundly provide some impacts on mass of polymer fed out from a tip of needle, morphology of the stretched jet (single or multiple jets), repulsion at the charged solution causing elongation (Tan *et al.* 2005). Surface tension viewpoint potentially does impress the resultant fibers in this case the formation of beaded-fibers or smooth fibers. For example, the images (Samples D0, D1, and D2) might represent an influence of surface tension from three different ranges of concentration made, even though other physical factor might affect as well. Fig.4.2-D0 seems to result in much bigger beaded-fibers due to low viscosity and surface tension compared with the other images, Fig.4.2-D1 and Fig.4.3-D2. Theoretically, when solution is stretched under the influence of highly electrified charges, solvent molecules spread out along with the entangled polymer chains, then solvent molecules of polymer solutions having low viscosity potentially tend to agglomerate each other due to surface tension, thus fabricating beaded-fibers probably as seen in Sample D0.

From the first group, chemically, PCL solutions are miscible in two distinct organic solvents. The former can be dissolved in chloroform:methanol, the latter is not miscible within chloroform:methanol due to the presence of the modified starch. Analysis of hydrophobicity of polymer solution utilized in this research has completely highlighted an important knowledge that starch acetate-compounded PCL is slightly hydrophobic, since starch acetate is slightly hydrophobic, and PCL is hydrophobic either. Such blended form was miscible then in formic acid. In addition, formic acid has dielectric constant higher than chloroform:methanol. Its dielectric constant is 58 (at 60° F) (<http://www.asiinstr.com/technical/Dielectric>). The elevation of dielectric constant is not dealing with the dropped off beads issued in. Starch acetate-compounded PCL actually fabricated more beads once formic acid was totally employed as solvent at high humidity. Nonetheless, a high dielectric constant of formic acid resulted is potentially inversely proportional with its evaporation rate. The

higher of dielectric constant of one solvent, the lower evaporation rate of certain solvent, while polymer is ejected out of a needle and solidified onto the grounded collector.

Beaded-fibers of starch acetate-blended PCL and starch acetate-coated PCL themselves could not be probably separated from the specific character of starch acetate, briefly the degree of substitution. The degree of substitution is possibly one of several factors beside process parameters and ambient parameter to fabricate more filaments than beads. Yang *et al.* has reported that three different degree of substitution of starch acetate, namely 1.1, 2.3, and 2.8 were able to generate fibers. Lower than such range might be problematic the fine fibers to deposit. As a matter of the fact that the acetylated starch acetate used here was DS equivalent to 1.6 (data can be seen in Appendix). As a conclusion, a medium value of DS could not be performed to manufacture beads-free at extremely high humidity.

The second and the four sections were performed in the same track by means coating of starch acetate. The diversity of two cases here is only the mixed-solvents employed. In the case of this Polycaprolactone intervened as a primary depositing surface, afterwards the secondary layer, starch acetate, was covered over the surface of PCL. While starch acetate was layered, PCL fibers might not be completely dry, this phenomenon probably touches off the accumulation of residual charges at the surface of both polymer fibers (the starch ester and PCL), potentially leading an influence to the resultant fibers. Such case is perhaps the reason why beaded-fibers emerged beside the effect of extraordinarily high humidity.

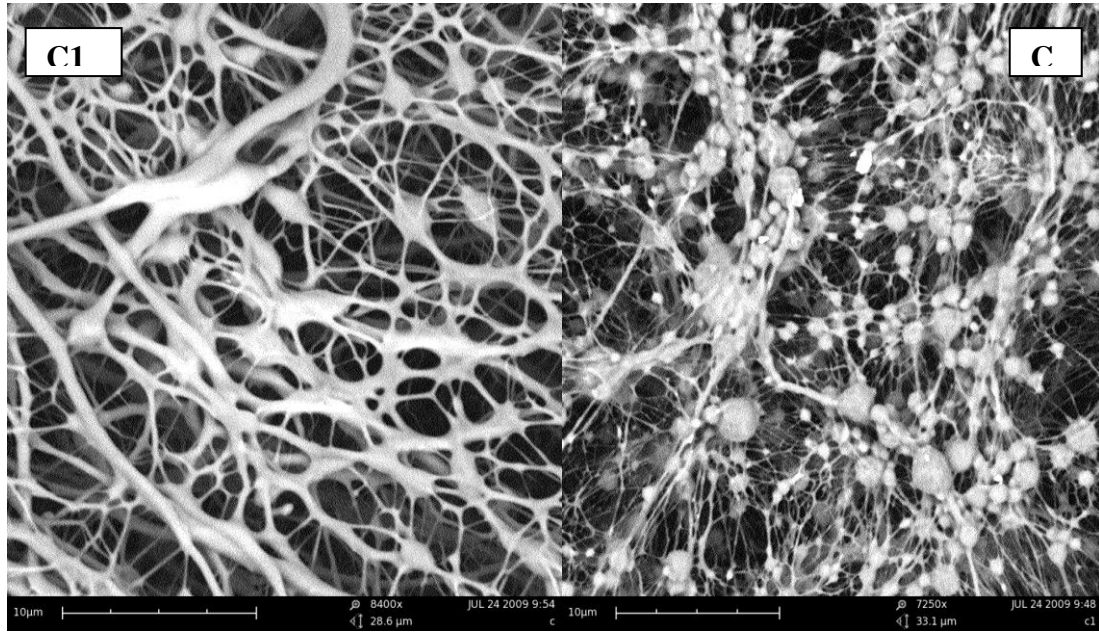
One of material parameters such as organic solvents employed does have prominently dissimilar features. In this first category, PCL was solubilized in chloroform:methanol, meanwhile the latter was dissolved in chloroform: DMF at certain range concentrations. In some literature, it is said that DMF has high dielectric constant compared to methanol. This towering dielectric constant is somewhat offset low evaporation rate. The collaboration among chloroform and DMF is slightly higher than other mixture in this case chloroform and methanol. According to the measured viscosity also has dominantly reported that mixture of chloroform:DMF brought high viscosity value out (see table 4.1), means that, DMF itself has low evaporation rate. However, evaporation rate is one of the several factors affecting the structures of electrospun fibers. In addition, the quality of solvents (denoted by  $R_{ij}^2$ ) in fact is able to prognosticate and is mathematically defined as  $R_{ij}^2 = 4(\delta_{d1}-\delta_{d2})^2 + (\delta_{p1}-\delta_{p2})^2 + (\delta_{h1}-$

$\delta_{h2})^2$  (where  $\delta_{d1}$ ,  $\delta_{p1}$ , and  $\delta_{h1}$  are three-dimensional solubility parameters of solvent;  $\delta_{d2}$ ,  $\delta_{p2}$ , and  $\delta_{h2}$  are those for polymer) (Liu J *et al.* 2008). From equation written, the lower  $R_{ij}^2$  due to solvent-polymer interactions, the better solvents used to dissolve polymer. By meaning that the quality of solvents either good or poor solvents can be potentially determined. Table 4.4 discloses lists of solubility parameters for solvents only (taken from Polymer Handbook written by Brandrup *et al.*).

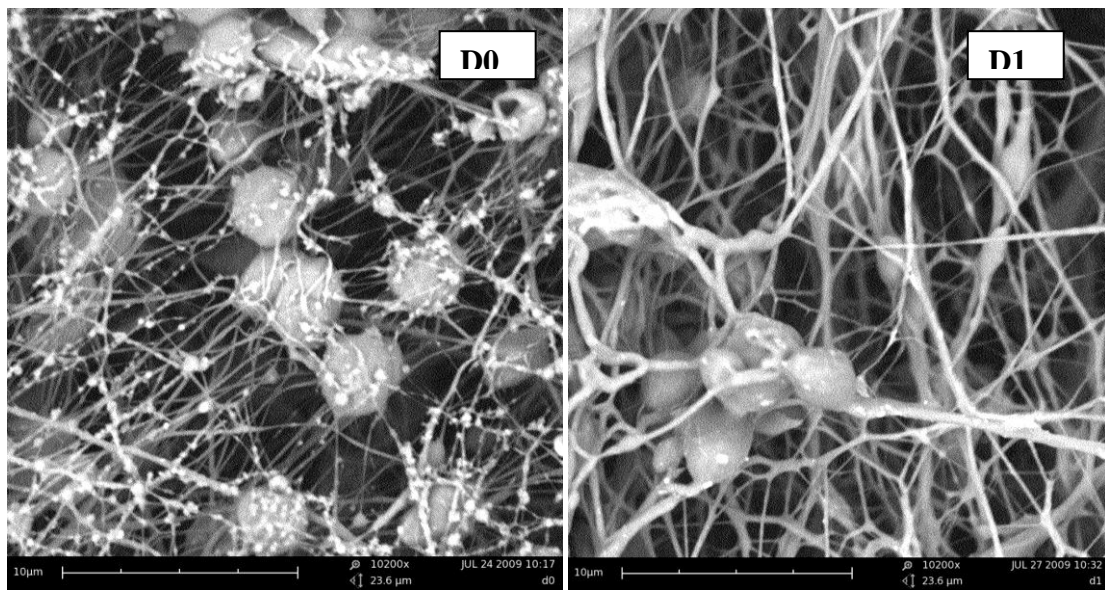
No	Solvents	Solubility parameters ( $\sqrt{\text{Mpa}}$ )		
		$\delta_d$	$\delta_p$	$\delta_h$
1.	Chloroform	17,8	3,1	5,7
2.	DMF	17,4	13,7	11,3
3.	Methanol	15,1	12,3	22,3

**Table 4.4** .The value of solvent solubility parameters

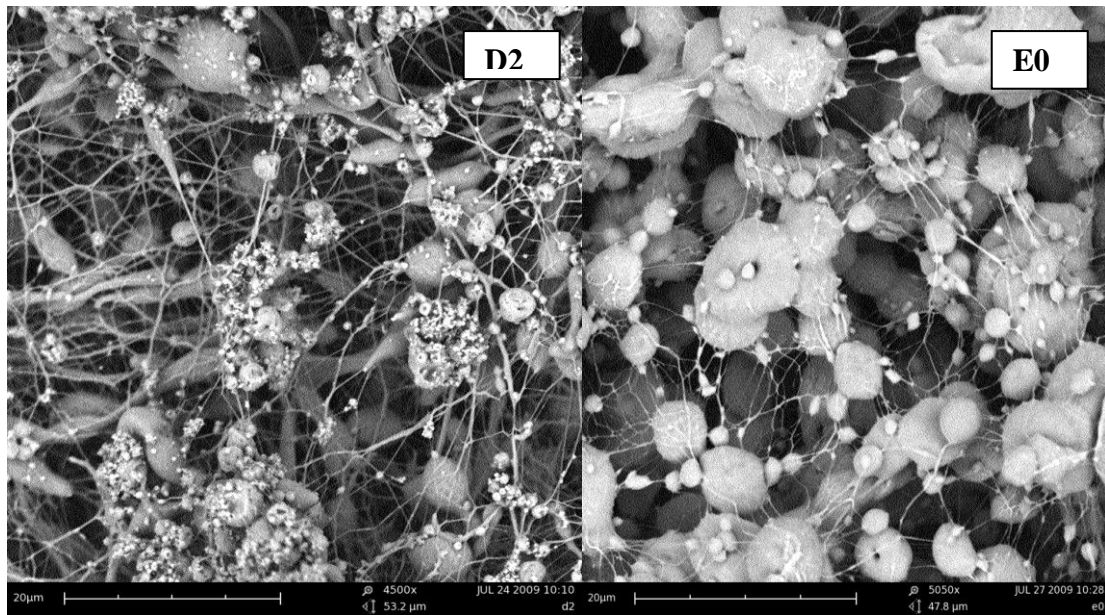
Based on Satish *et al.*  $R_{ij}^2$  is recognized as common value to determine the quality of solvent. It depends on two solubility parameters, one is taken from solvent solubility parameters and the other one is dominantly gained from polymer. As a matter of fact, polymer used was akin specifically PCL, dissolved in two different solvents. We can probably modify the calculation exerting Hansen-Hildebrand's equation as follows:  $\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2$ . Hansen-Hildebrand's equation computed ultimately illuminates that solubility parameters of chloroform:DMF is slightly lower than chloroform:methanol, means that chloroform:DMF is good solvents for PCL and is possibly better than other one. Unfortunately, we could not see clearly the effect here, since polymer solutions consisting of PCL were spun at high humidity.



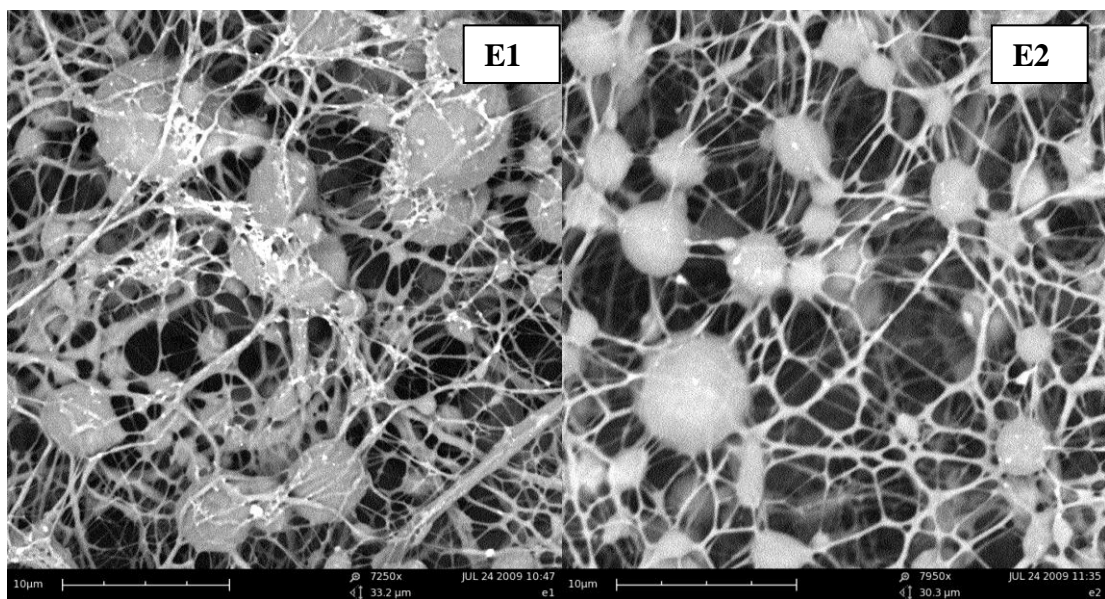
**Figure 4.1** C1. Polycaprolactone (PCL) in chloroform: methanol (3:1). C. Starch acetate-blended PCL (total 15 % wt of solution).



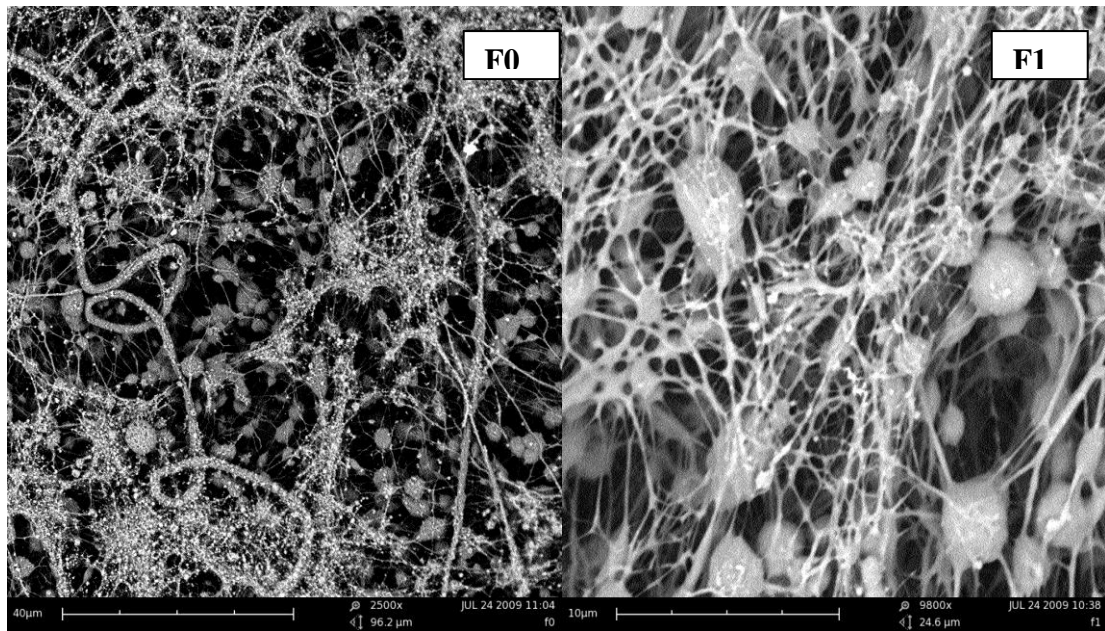
**Figure 4.2** Twenty percent of starch acetate and 10 %, 11 %, and 12 % wt exhibited in D0 and D1 respectively. Both D0 and D1 were of PCL samples dissolved in chloroform:methanol.



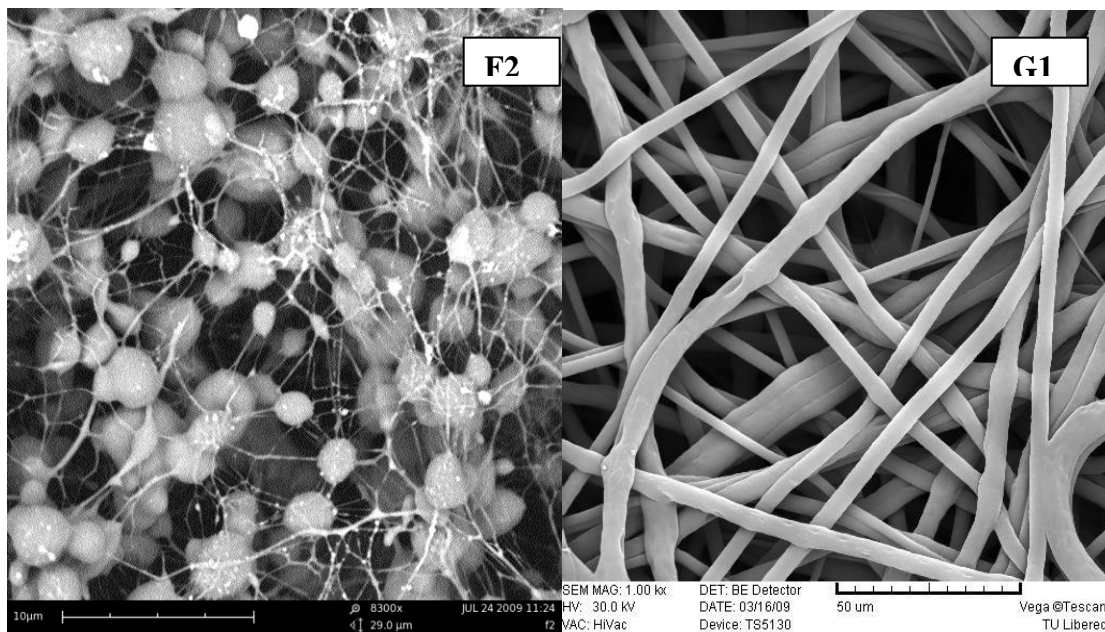
**Figure 4.3** From left to right: D2. It consisted of 20 % wt. starch ester coated with PCL. E0. A pristine PCL (10 % wt) was dissolved in chloroform:DMF



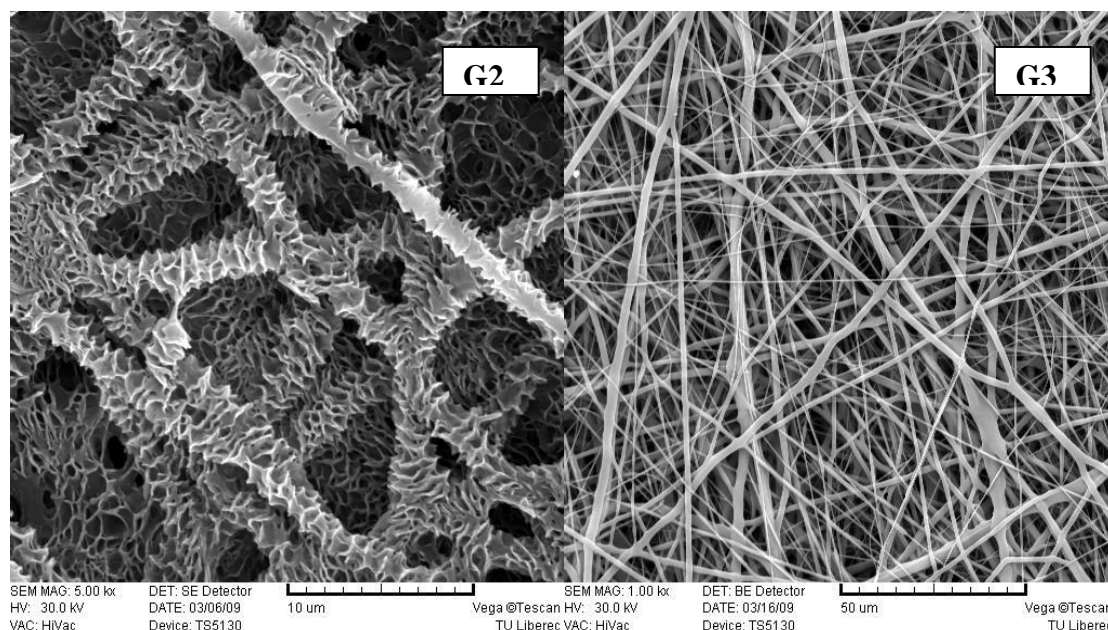
**Figure 4.4** The PCL solutions containing 11 % and 12 % wt depicted in E1 and E2, respectively. These samples were solubilized in chloroform:DMF



**Figure 4.5** Fifteen percent of starch acetate was coated with 10 % and 11 % wt of PCL, as shown in F0 and F1, respectively



**Figure 4.6** From left to right: F2. It contained 12 % wt of PCL impregnated with 15 % wt starch ester. G1. Starch particles-blended PCL (in chloroform:methanol ( starch particles were not in such image, only fibers given by PCL).



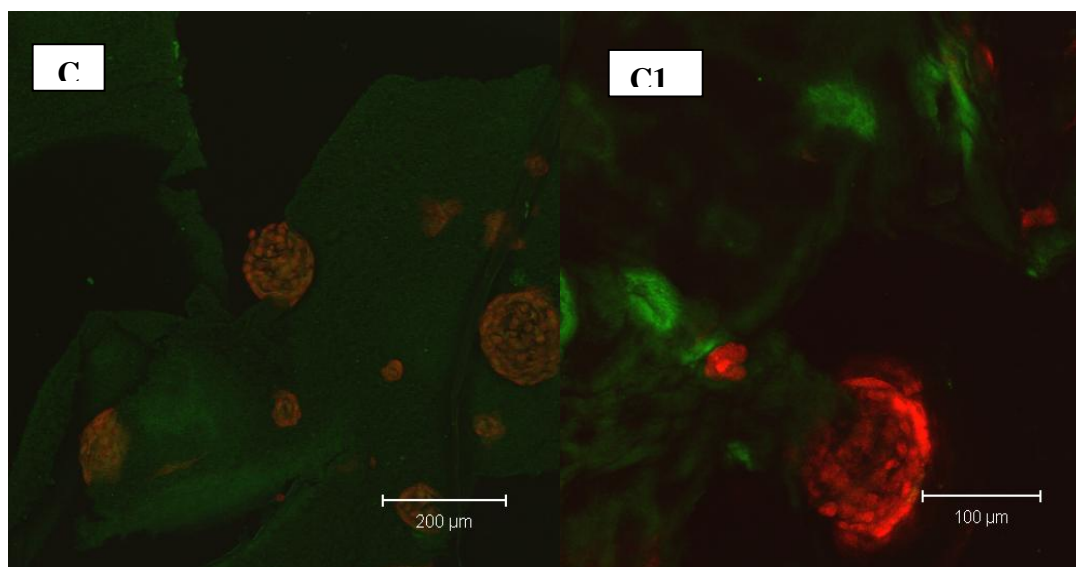
**Figure 4.7** From left to right. These entire fibers outcomes were carried out under humidity of 45 %, except G2, since it was done at extremely low humidity less than 20 %. G2 and G3 were of starch particles-compounded PCL in chloroform:DMF (no starch presented), starch particles-compounded PCL in chloroform:DMF at average humidity ( $\leq 40\%$ ).

## 4.2 Tissue engineering

Two variants of histological staining solutions, propidium iodide and invitrogen, are commonly used to examine the study of microscopic behavior of cells. As mentioned in chapter 3, cell cultivation part, propidium iodide was performed on Samples C, C1, D0, D1, and D2. Another histological staining solution, invitrogen, was utilized on the rest samples, namely E0, E1, E2, F0, F1, F2, and PCL prepared by the Institute of Clinical and Experimental Medicine.

The depicted images (green color was polymer membranes, and the red one was a group of chondrocyte cells) of Samples C1 and C, exhibit dominantly that cells were not distributed or migrated over the surface of hydrophobic polymers. In other words, cell-cell interactions were remarkably more robust than cell-substrate interactions. Chondrocyte cells tried to communicate by acting as spheroids on C and C1, as seen in Fig. 4.8. Somewhat similarly, figure 4.9 (D0,D1, and D2) also shows the same behavior as previous images. In general, Samples E0, E1, E2, F0, F1, F2, as well as F3 demonstrated that the defined cells tried to spread over the surfaces of scaffolds rather than interact amongst them, as seen in Fig. 4.9-E0, 4.10, and 4.11. It

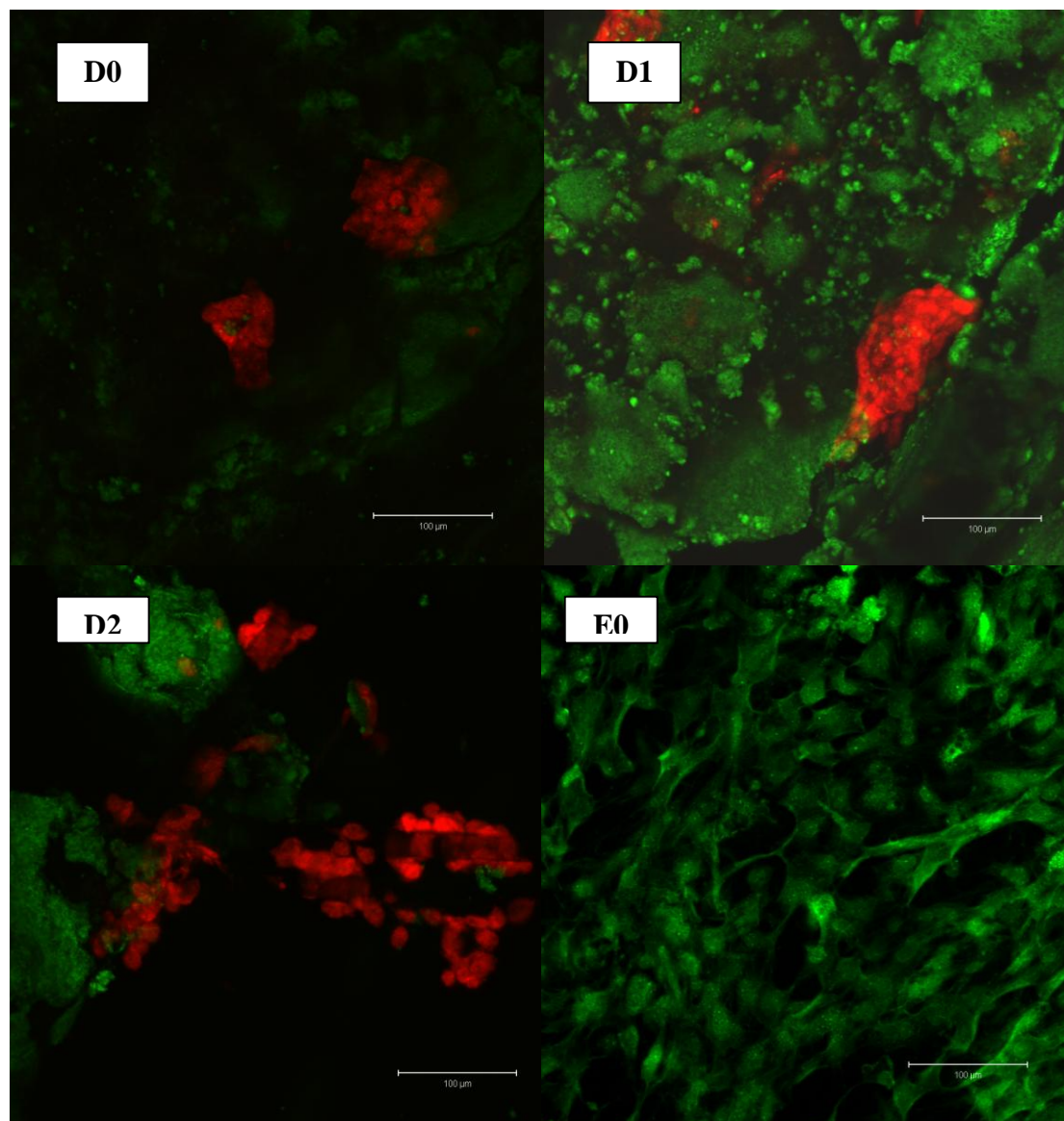
is rather difficult to examine that both of histological staining solutions had a deep impact in cellular behaviors.



**Figure 4.8** The cells preferred to interact amongst them rather than attach onto starch acetate blended PCL (C) and a pristine PCL scaffold (C1), respectively

MTT test is potentially needed to perceive proliferation rate of the cells cultured on the surface of scaffolds and probably sustains the images captured by a confocal microscope. The absorbance of starch acetate blended PCL (C1) and a pristine PCL scaffold dissolved in chloroform:methanol was respectively  $0.078 \pm 0.012$  and  $0.014 \pm 0.005$  on day 1. On day 7, the absorbance given was slightly lower than the first day of cultivation,  $0.074 \pm 0.027$  and  $0.014 \pm 0.013$ , as seen in Fig.4.12. In general, based on an MTT assay, the scaffold made up of starch acetate blended PCL demonstrated a positive response in cell viability slightly better than PCL scaffold solubilized in chloroform:methanol. Thus, the scaffold made up of starch acetate blended. In contrast, figure 4.13 shows that a sample containing 10 % PCL coated with 20 % (D0) exhibited an increase in cell viability from day 1 to day 7,  $0.035 \pm 0.040$  and  $0.156 \pm 0.123$ , respectively. Such sample was statistically better in promoting cell viability than the rest of samples in the same group, D, namely D1 and D2. Thus, the small amount of PCL scaffold impregnated with a higher acetylated starch slightly significant altered the primary step of cellular activities, cell viability. Then, the absorbance (570 nm) of a pristine PCL (sample E0) and starch acetate-coated PCL (sample F0) were  $0.080 \pm 0.013$  and  $0.084 \pm 0.019$  on day 1. On day 7, the cell viability of both matrix scaffolds, PCL (sample E0) and acetylated starch

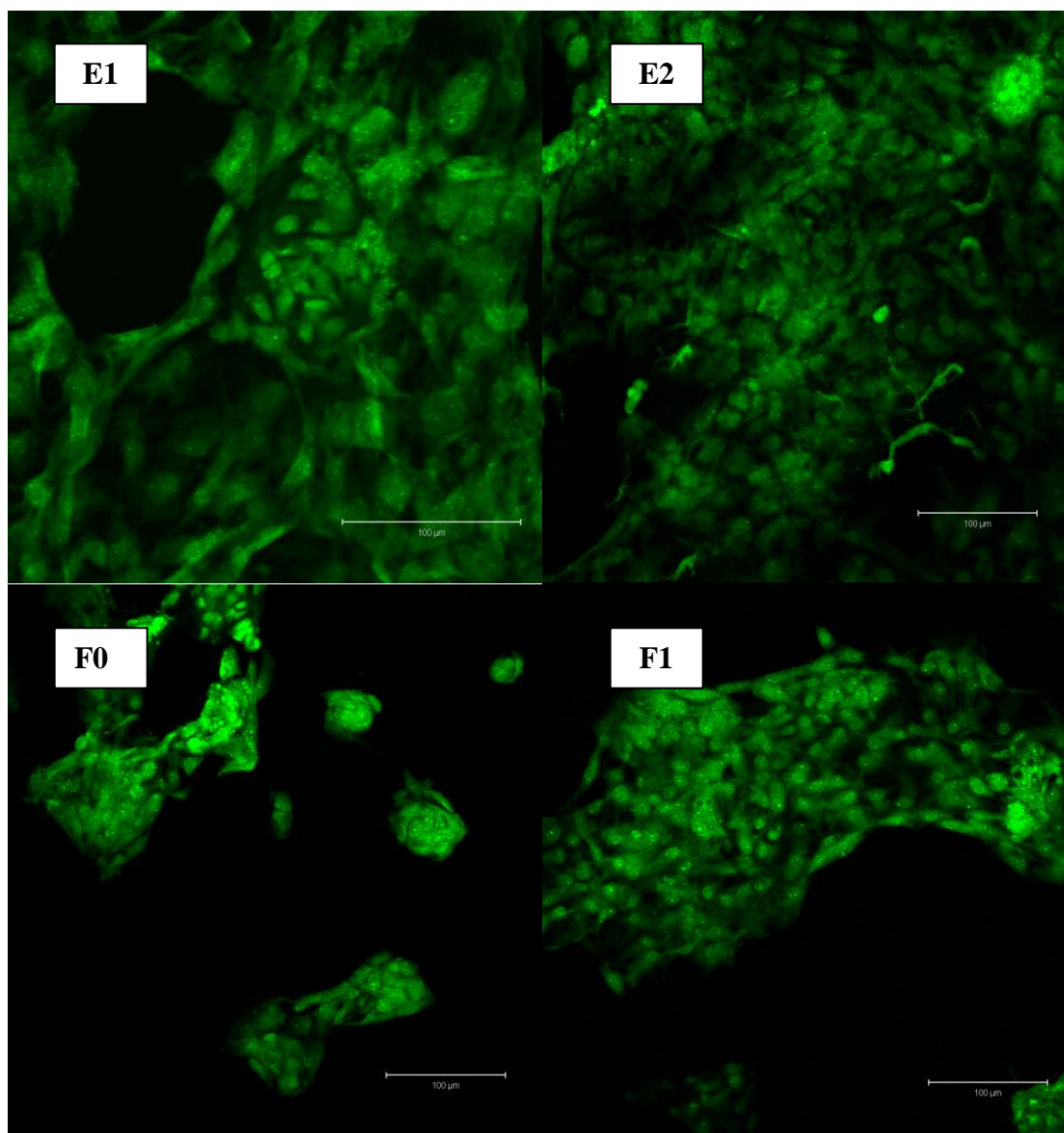
coated PCL (sample F0), statistically increased into  $0.253 \pm 0.059$  and  $0.375 \pm 0.168$ , respectively. One should bear in mind the viability of chondrocytes slightly improved on the surface of starch ester-layered 10 % (w/w) PCL as compared with a 10 % wt PCL without starch acetate, as seen in Fig.4.14



**Figure 4.9** The chondrocytes slightly preferred spreading onto polymer based scaffolds (D0, D1, and D2) than interacting amongst them. The cells tried to adhere onto the surfaces of scaffolds in spite of forming colonies. The cells were well-adhered on the surface of PCL scaffold (E0).

By the time PCL concentration was increased, and then impregnated with starch ester on its surface, no influence was statistically achieved in cell viability after comparing with the pure PCL though. On the other hand, starch acetate could influence cell viability with less amount of PCL scaffold, showing better result in enhancing the viability of the defined cells than a 10 % wt pristine PCL, but the coating process of starch acetate with higher concentration of PCL (11 % and 12 % wt) would

statistically demonstrate similar performance in the viability of the chondrocytes to the pure PCL consisting of 11 % and 12 %wt, respectively. In general, one needed to underline is starch acetate coated PCL had similar performance in cell viability as compared with the most widely known matrix scaffold, PCL. Additionally, at 7 days, a tissue control, CHP, declined its absorbance ( $0.288 \pm 0.053$ ), giving lesser performance as the rest of other substrates.

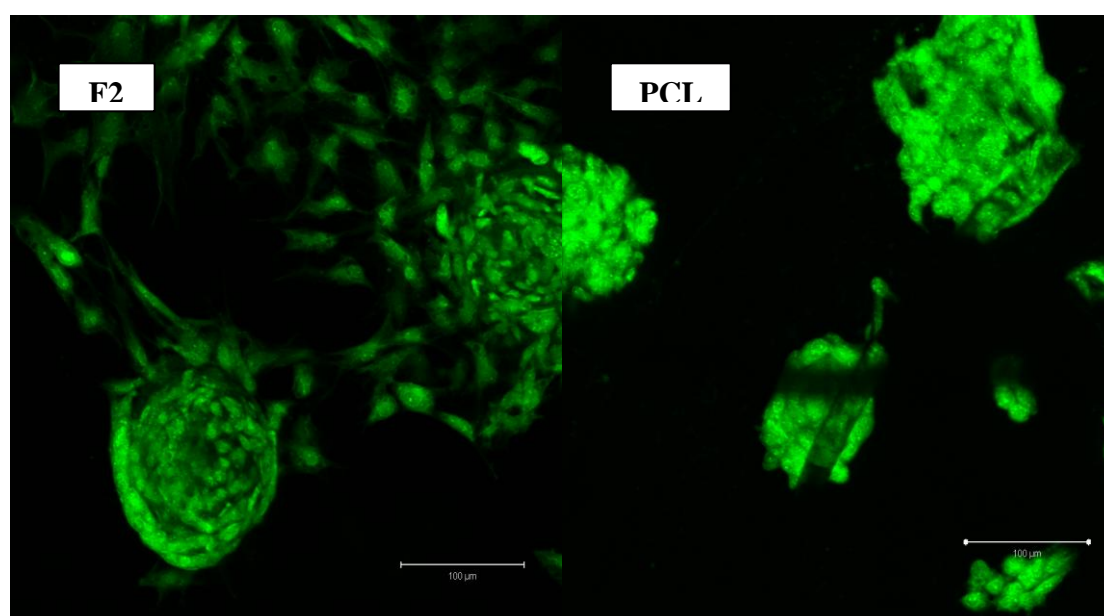


**Figure 4.10** In general, the cell-surface interactions were stronger than cell-cell interactions (E1, E2). Acetylated starch coated PCL (PCL dissolved in chloroform:DMF) (F0,F1) was much better in cell attachment than starch coated PCL (PCL is miscible in chloroform:methanol).

The 3D dimensional matrices can be prepared by electrospinning. They act as artificial ECMs (Extracellular Matrices) over which the defined cells spread extensively and homogenously. Polymers that were investigated to develop

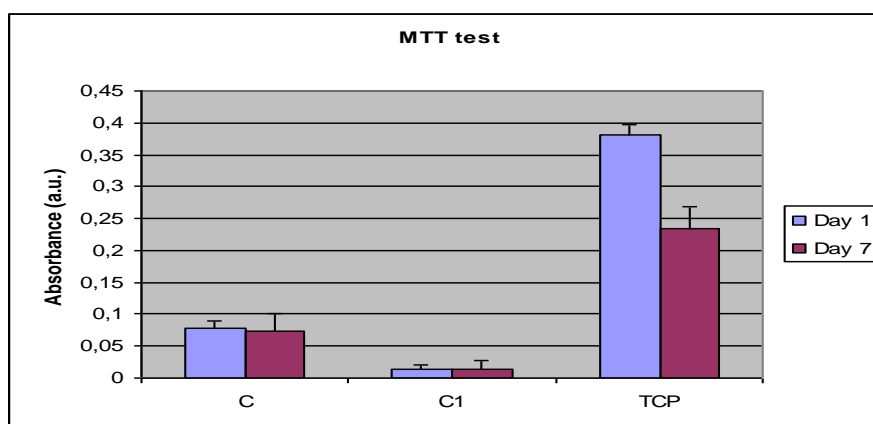
biodegradable nanomats include natural, synthetic, and a combination thereof (Ashammakhi *et al.* 2006).

In this work, two sorts of biodegradable polymers were utilized as potential scaffolds for tissue engineering, PCL and starch acetate coated PCL. Obviously, tissue-engineering scaffolds are manufactured to serve as temporary, cells' mediator and to guide 3D tissue formation via cellular activities starting from cell adhesion [Goldberg *et al.* 2007]. However, cell-surface interactions play a critical role for selection biomaterials applied as basement membranes in tissue engineering. Furthermore, the cell-polymer configuration will be exploited to allow-in gas exchange and diffusion of nutrients required by specific cells (Zund *et al.* 1999).

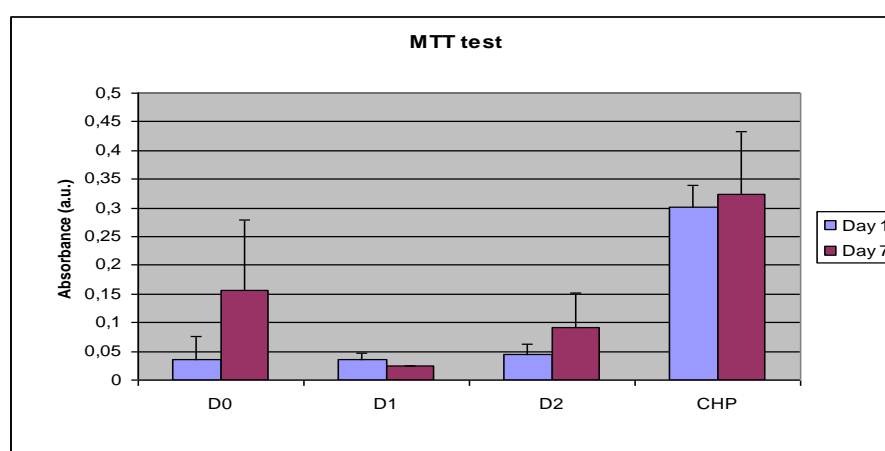


**Figure 4.11** The cells tried to attach over the surfaces of scaffolds made up of starch acetate coated PCL (PCL dissolved in chloroform:DMF) (F2) and PCL prepared by the Institute of Clinical and Experimental Medicine, respectively.

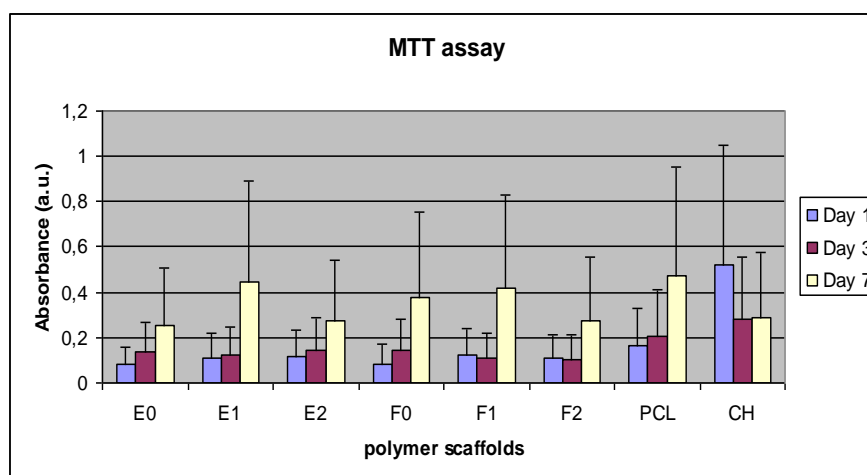
Chen *et al.* 2007 investigated a novel study about cell-surface interactions of the specific cells on hydrophilic/hydrophobic biomaterials. They demonstrated that such cells adhered and grew preferentially on hydrophilic biomaterials. Another report which emerged, Chang and his co-workers performed the investigation of the cell-matrix interactions. They further observed that the osteoblastic MG-63 cells reduced their proliferation and elevated apoptosis on the hydrophobic surface polymers than the hydrophilic surface polymers.



**Figure 4.12** MTT assay of chondrocytes cultivated on starch acetate blended PCL scaffold (C1), a pristine PCL (C), and a tissue control, CHP.



**Figure 4.13** The results of MTT assay of chondrocytes seeded on D0, D1, D2, as well as a tissue control from day 1 to day 7.



**Figure 4.14** MTT assay of chondrocytes cultivated on PCL scaffolds (E0, E1, and E2), starch acetate coated PCL (F0, F1, F2), PCL, and a tissue control, CHP.

By contrast, in this work, the chondrocyte cells were generally well-spread over the scaffold surfaces which were made up of hydrophobic biomaterials, as

depicted in Fig. 4.9-E0, 4.10, and 4.11, respectively. In addition, starch in a pristine form is a hydrophilic polymer but, it chemically alters its character after modifying into a modified form, an acetylated starch. Probably, cell-matrix scaffold interactions are a very sophisticated phenomenon, it is not quite clear which property, hydrophilic or hydrophobic will be favorable for cell attachment, proliferation and other complex cellular activities (Lee *et al.* 1994).

This study presents cell viability on novel combined polymer scaffolds. As has been indicated previously, the electrospun beaded fibers (D0, D1, D2, F0, F1, as well as F2 resulted from 20 % wt starch acetate coated PCL (PCL dissolved in chloroform:methanol) and 15 % wt. starch acetate PCL (PCL dissolved in chloroform:DMF), respectively, and the last group consisted of E0, E1, and E2 made up of a pristine PCL demonstrated no significant differences in cell viability amongst them except the scaffolds made up of starch acetate PCL and PCL were statistically slightly distinct. The MTT test is widely used for *in vitro* measurement of the metabolic activity and proliferation of cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] is reduced to purple formazan by mitochondrial dehydrogenase in cells indicating normal metabolism. MTT is cleaved by all living metabolically active tested cells. Activated cells are capable of producing more formazan than resting cells, allowing the measurement of activation even in the absence of proliferation (Mosmann 1983).

The results given by an MTT assay did not allow us to select which of the scaffold materials is best in this work. The native starch, either in a modified form or in its blended forms with polyester groups, is still poorly investigated as a promising scaffold in tissue engineering. From this study, starch acetate positively provided an increase of cell viability from day 1 to day 7 when either it coated or blended with PCL scaffold. However, in tissue-engineered scaffolding, most the defined cells require attachment to a solid substrate for viability, growth, migration, as well as more complex processes such as differentiation. Moreover, the function and behavior of adherent cells are dependent on the characteristics of the solid substrates, particularly its adhesiveness [Schoen FJ *et al.* 2004, Piskin *et al.* 2007]. Yet, some of the requirements for the design and production of an ideal scaffold have to be taken into account, including the use of non-toxic, biodegradable, biocompatible materials, which also do not cause an inflammatory response (Edwards *et al.* 2004, Coutu *et al.* 2009). PCL is a hydrophobic polymer which is frequently used as a matrix scaffold

in cartilage tissue engineering; in contrast, starch acetate –blended and -coated PCL are a novel scaffold material, this work positively provided a good response in the viability of chondrocytes similar to PCL though. A challenging work will be required to reveal their behaviors more profoundly such as cytotoxicity assay, degradation kinetic.

## ***5 Conclusions and Tasks***

Conclusions are divided within two main categories in question, electrospinning and tissue engineering.

### ***5.1 Electrospinning conclusions***

1) Overall, this work was potentially strongly influenced by the elevated humidity which might be poorly investigated. The electrospinning process of PCL and its blended design optimally performs at average humidity, thus leading less beaded-fibers resulted (see some figures in Chapter 4 particularly the resultant fibers of two different scopes of humidity, medium and high). During electroprocessing the humidity was extraordinarily high; generally the electrospinning process of PCL and starch acetate is conducted in the range of 30 – 40 %. Thus, it is not possible to achieve well-defined filaments at elevated RH (>40%). Hence, a continuing work might be done to reveal the influencing ambient parameter. As a conclusion, starch acetate-blended PCL, PCL dissolved in a variety of mixed-solvents, as well as starch ester coated PCL are able to optimally electrospun at humidity less than 50 % or possibly in the range 30-40 %.

2) A mixed-solvent such as chloroform:DMF is good solvent for the electrospinning process compared with chloroform:methanol, based on Hansen-Hildebrand's equation which provides the quality of solvents in electroprocessing. Perhaps, such character could be also matched due to the fact that the boiling point of DMF is much higher than methanol has, and the elevated boiling point sometimes takes the time to evaporate. Such physical property was potentially sustained by the high relative humidity, thus, even though chloroform:DMF is good solvent for mostly PCL in electrospinning process, the finer fibers will not possibly achieved.

### ***5.2 Tissue engineering conclusions***

1) However, cell-surface interactions play a critical role for selection biomaterials applied as basement membranes in tissue engineering. In this work, the chondrocyte cells were generally well-spread over the scaffold surfaces which were

made up of hydrophobic biomaterials such as starch coated PCL (PCL dissolved in chloroform:DMF), PCL scaffold dispersed in chloroform:DMF except PCL scaffold dissolved in chloroform:methanol and starch acetate blended PCL, though an MTT assay demonstrated statistically that starch acetate blended PCL was much better in promoting cell viability than PCL scaffold.

2) As has been previously noted that an MTT test is obviously useful to quantitatively determine which polymer scaffolds will influence cellular activities starting from its primary steps, cell viability. Samples containing less amount of a pure PCL scaffold (10 % wt) impregnated with starch acetate played a crucial role in promoting the viability of chondrocytes. They showed much better than other variants containing higher concentration of PCL scaffold, 11 % and 12 % wt, coated with starch ester. They were also slightly quantitatively much better than pristine PCL scaffold. As a conclusion, in general, starch acetate and its either blended or coated designs have the same performance in sustaining cell viability as PCL scaffold. A series of strategies will be surely needed to further improve their characters to be similar to the widely used control scaffold, TCP.

### ***5.3 Tasks for Future Work***

In fact, several recommendations in this work constitute an important key to reveal more detail either in the electrospinning process or tissue engineering case.

#### ***5.3.1 Part of electrospinning***

1) By using the same polymer solutions, starch acetate-incorporated PCL, pure-PCL dissolved in two types of mixed-solvents, as well as starch ester coated PCL, the electrospinning setup should be adjusted properly, particularly in case of the ambient parameter. Each of groups can be set up on various ranges of the exact humidity, for instance in the range of 20 up to 60. From this manner the relative humidity can be completely examined and how far it does affects the resultant fibers in a variety of polymer solutions. The remainders of process parameters, namely NCD, flow rate, internal needle diameter, can be similarly set up.

2) The electrostatic spinning process of starch ester did not occur optimally. Numerous ranges of DS of starch ester should be initially examined, and then electrospun at either low or medium humidity, since at extremely high relative humidity did not emerge more fine fibrous mats.

3) The choosing of solvent or mixed-solvents used for starch ester should be taken into account to improve and result in fine fibers. The author has painstakingly striven with a variety of organic solvents, such as pyridine, acetyl acetone. The modified or acetylated was completely miscible in such solvents formulated. Yet, the solubility of starch ester is further strongly dependent on DS given. Once starch ester-blended PCL solubilized in pyridine completely, after stirring at 40°C overnight, electrospun at elevated humidity, and then fabricated dust-like structures. Additionally, future work is necessarily carried to uncover totally what have significantly caused scales-like structures on PCL fibers (shown in extra figure).

### ***5.3.1 Part of tissue scaffolding***

This part is totally similar with new phenomena in the electrospinning process. However, it is apparently needed to explore what is the prominently primary factor causing cell-cell or cell-surface interactions.

1) The surface property of biopolymers can be modified after electrospinning to alter either hydrophobic character or hydrophilic into new surface property becoming slightly hydrophobic and hydrophilic. The selection of chemical compound containing some functional groups should be taken into account. Several techniques have been explored to alter the initial surface property, such as using fluorescent proteins, radiation grafting, and etc. Ma *et al.* demonstrated that the surface modification of electrospun fibers could improve the spreading of the cells, following other cell activities, example cell adhesion, proliferation. Additionally, Park and his co-workers have demonstrated that biodegradable polymers such PGA, PLGA can be chemically or physically modified into hydrophilic one to encourage higher cell adhesion, attachment. The surface modification is chemically facilitated by incorporating of Arg-Gly-Asp (RGD) or other bioactive protein to be taken up by the scaffolds surface. Additionally, the surface modification can physically carried out using the plasma method.

2) As has been previously noted that the surface property, namely hydrophobic or hydrophilic one, is primarily important to determine either cell-cell or cell-matrix interactions, as seen and described in chapter 4, including some figures and MTT assays. The previous information reported by Lee *et al.* 1994 has obviously elucidated that cell-surface and cell-cell interactions are sophisticated phenomena, yet it is feasible to explore more detail regarding such behavior. We can perform the behavior cells that will be cultivated onto two distinct properties, hydrophilic and hydrophobic, and further analyze with one of numerous growth factors, such as Fibroblast Growth Factor-1 (FGF1), Bone Morphogenetic Proteins (BMPs), etc, and then assess the signaling pathway of growth factor used by using the protocol method so called activated Ras and Rac assay. As we know that, activation of Ras signaling enhance cell behaviors of cells, for instances cell growth, cell differentiation as well as viability. The protocol previously described has demonstrated by Chang *et al.* 2005. Chang *et al.* further reported that the surface property on which cells embed is associated with growth and apoptosis of the cells, precisely the osteoblastic cells. It is certain that insufficient of Ras signaling activation in FGF1, due to adhesion of chemical signaling pathway on the hydrophobic surface results in low Akt and ERK activation. ERK is the abbreviation of Extracellular signal-Regulated Kinases, and Akt itself commonly mentioned as serine/threonine kinase, is broadly utilized to mediate the majority of signaling pathways between the cells and the surface of scaffolds. Hopefully, this assessment for future work will possibly explore cell-surface cell-cell interactions on hydrophobic and hydrophilic character of biopolymers used in this work for instance.

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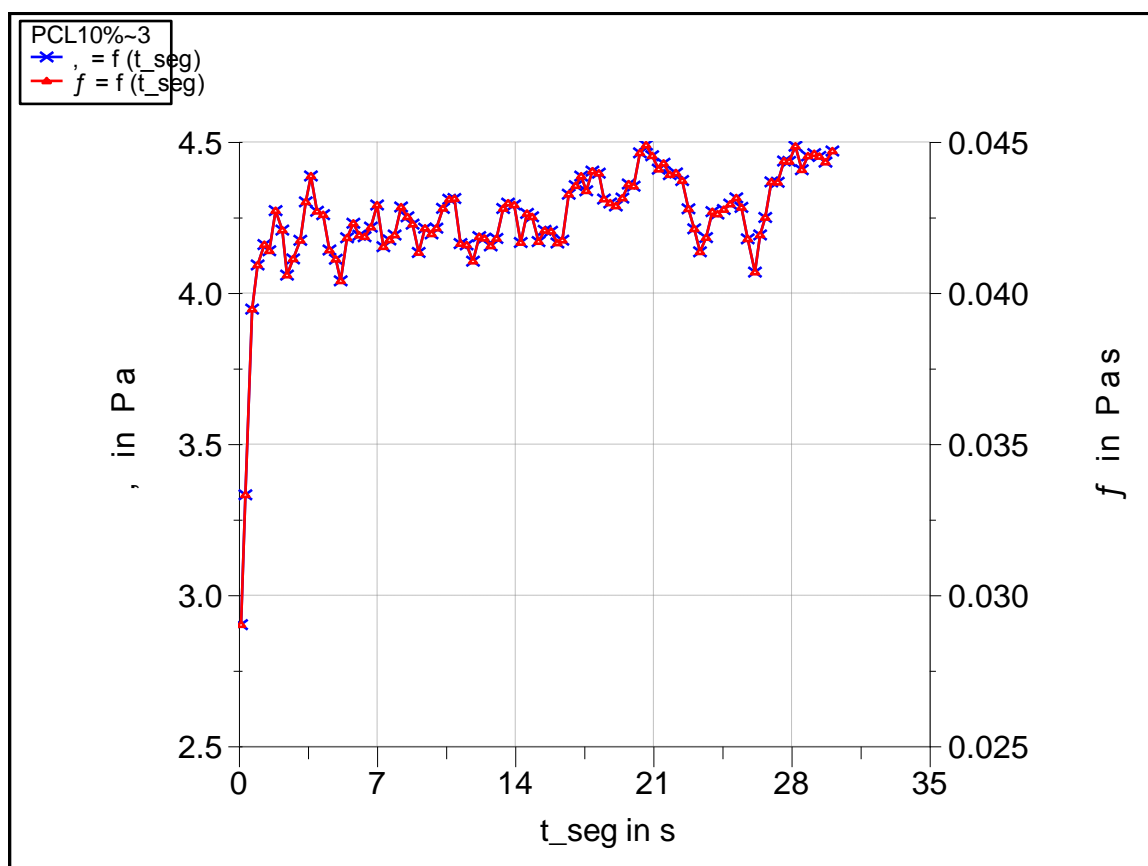
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## 7. Appendix

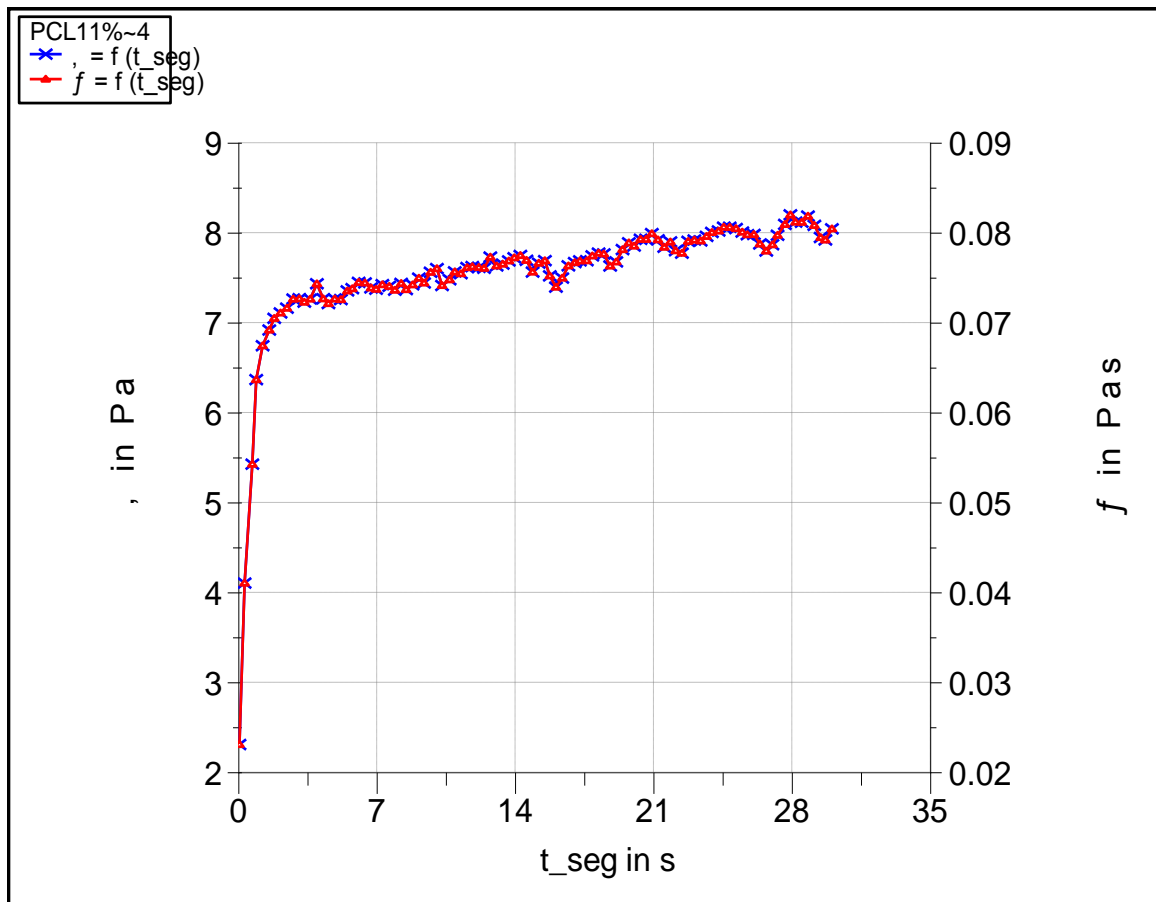
A. The results of measuring viscosity by RotoVisco1 are given as follows:

### 7.1 The viscosity graphs of PCL dissolved in Chloroform:Methanol

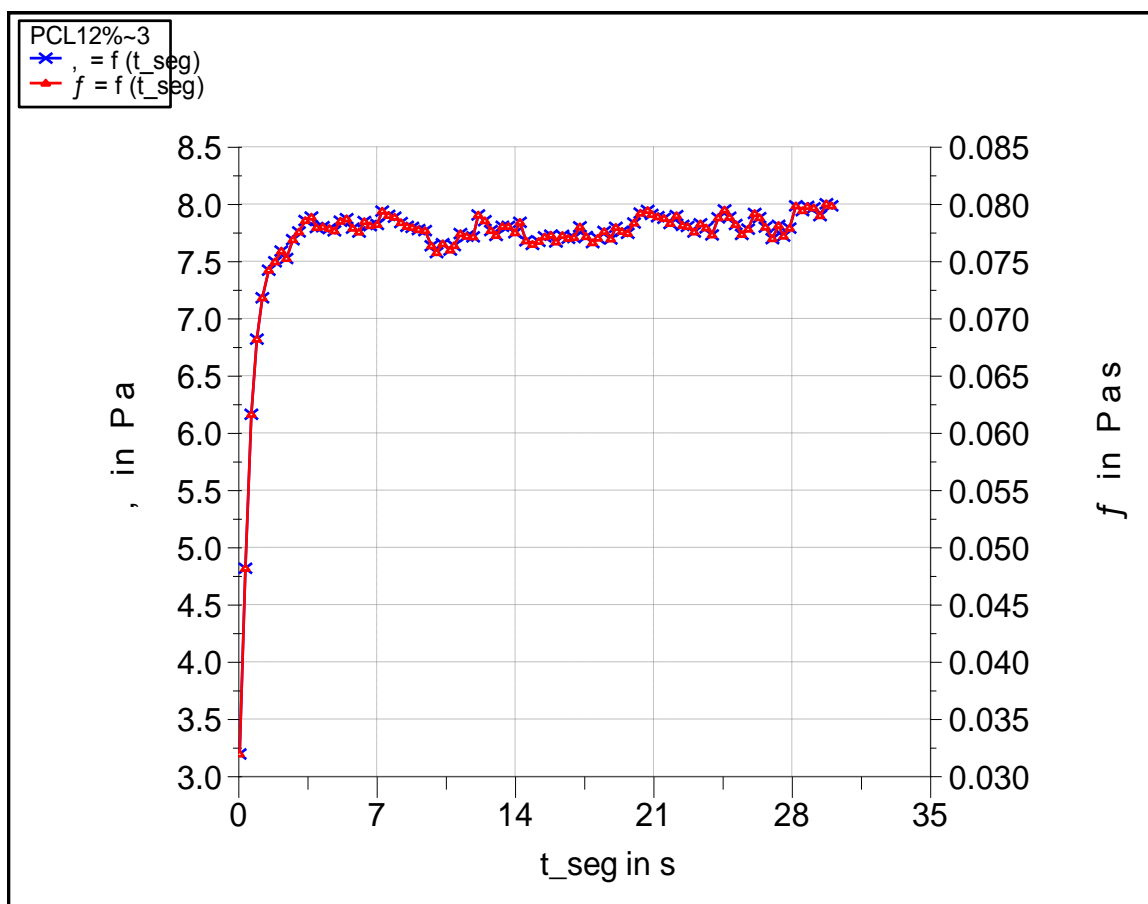
A 10 % wt PCL



An 11 % wt PCL

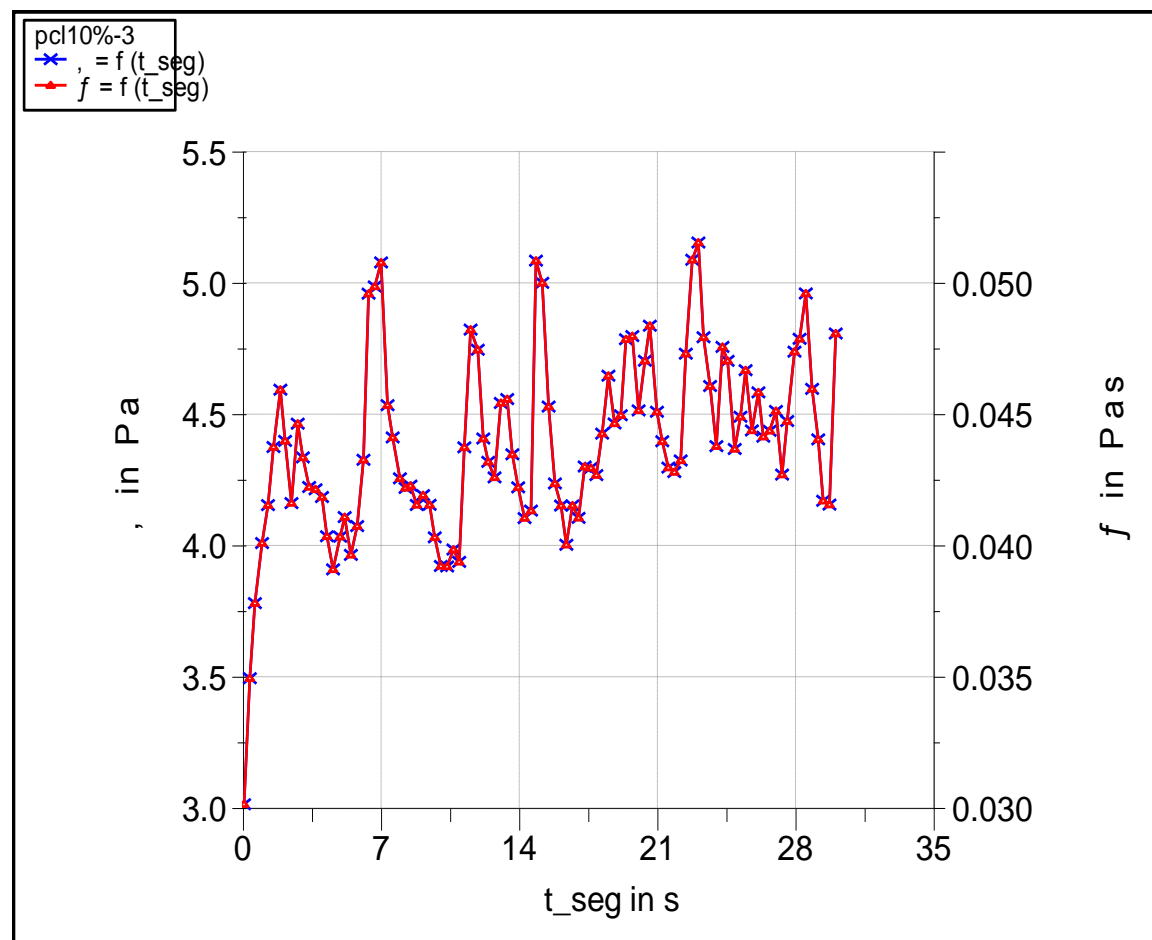


A 12 % wt PCL

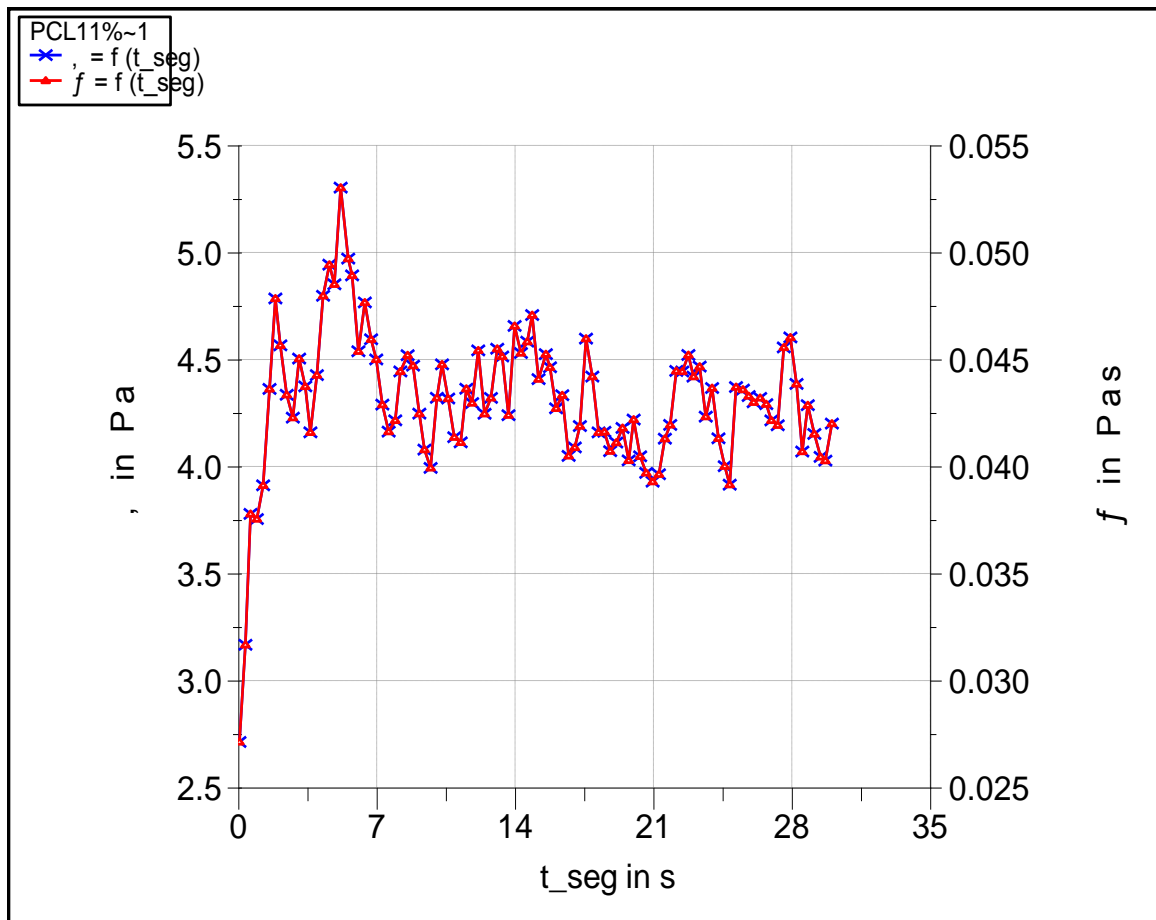


## 7.2 The viscosity graphs of PCL dissolved in DMF:Chloroform

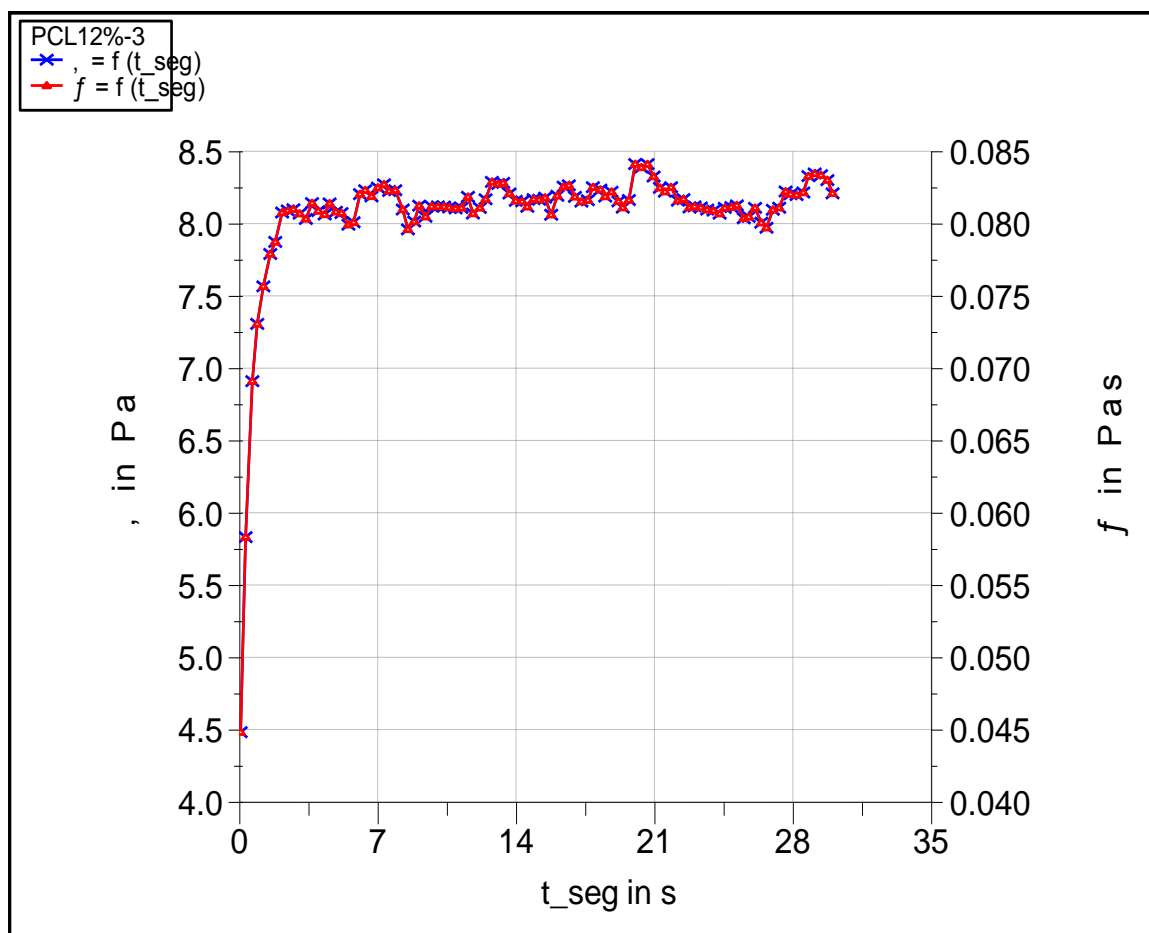
A 10 % wt PCL



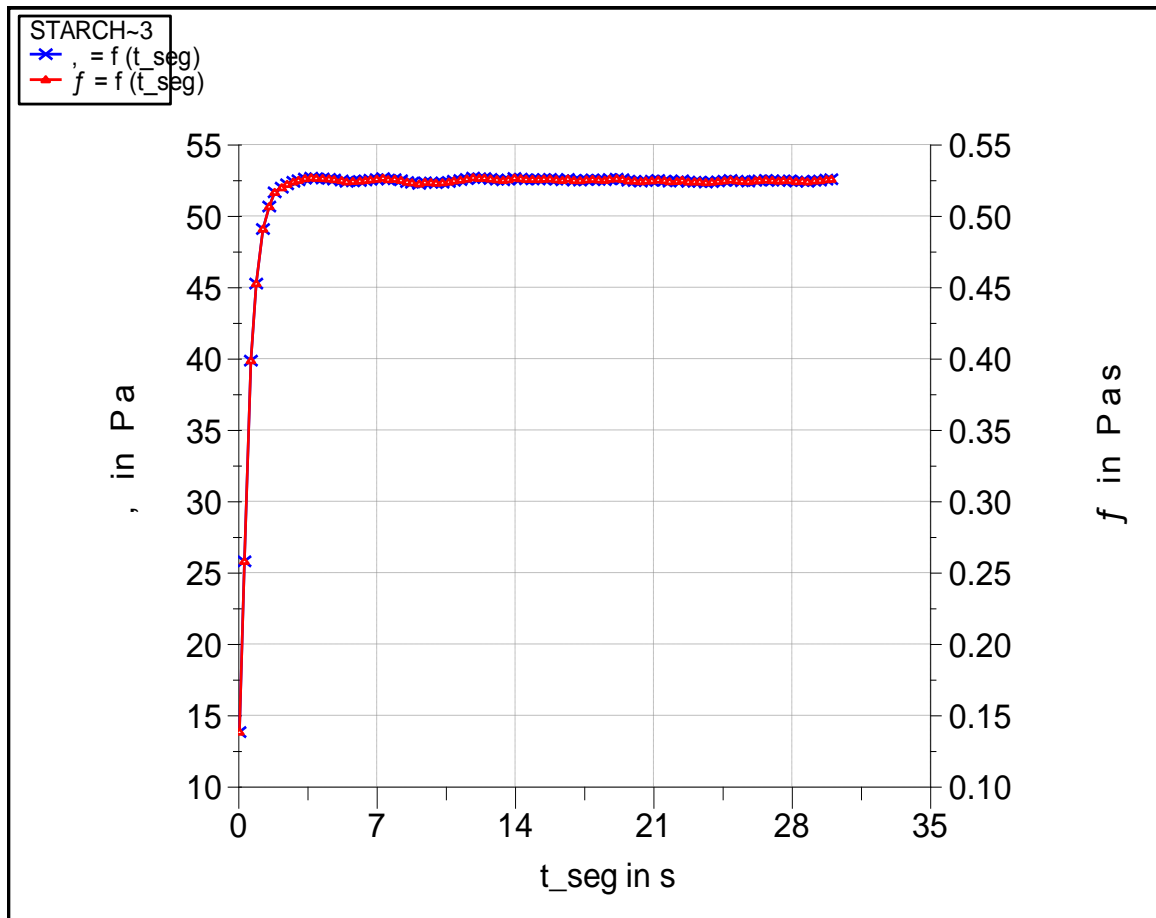
An 11 % wt PCL



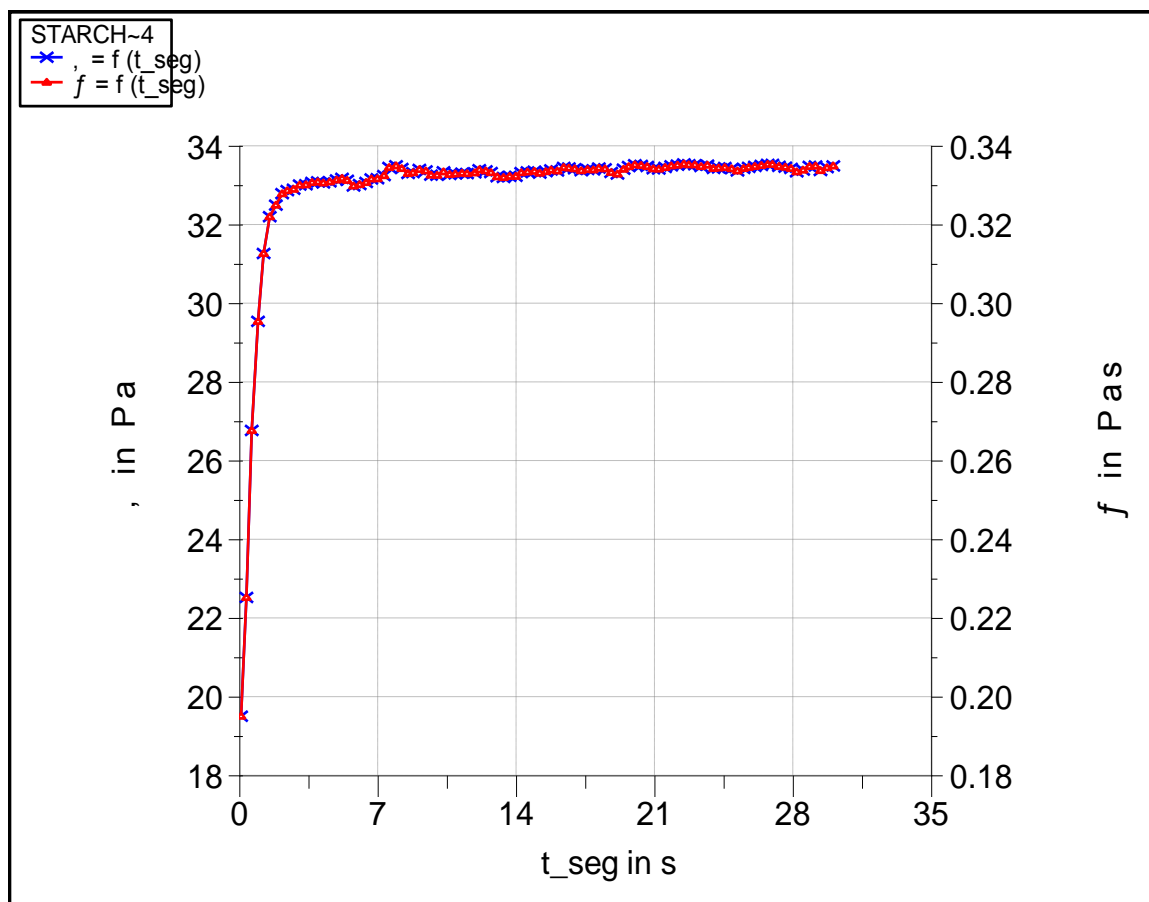
A 12 % wt PCL



### 7.3 The viscosity graph of a 15 % wt of starch ester



#### 7.4 The viscosity graph of a 20 % wt of starch acetate



## B. The determination of DS of starch ester

Degree of substitution of starch acetate

Determination of DS of starch acetate was carried out by titration method which involves the fundamental scope of basic hydrolysis and then titrate the excess alkali. The attempt of determining starch acetate's DS was performed twice due to the limited stock. The protocol used here followed several salient points in the article reported by Wang Xu *et al.* 2008. The following results are mentioned as follows:

The concentration of HCl = 0.495 N

The concentration of NaOH = 0.500 N

Indicator of titration = Phenolphthalein (pp)

The volume of first blank = 24.1 mL

The volume of second blank = 23.9 mL

The volume of starch acetate required to neutralize NaOH = 16.6 mL (first sample)

The volume of the acetylated starch needed = 17.4 mL (second sample)

The formula applied to compute DS is:

$$A\% = \frac{(V_0 - V_n) \times N \times 43.10^{-3}}{M} \times 100\%$$

$$DS = 162 \times \frac{A\%}{300 - 42 \times A\%}$$

$V_0$  = the volume of 0.485 N used to titrate the blank

$V_n$  = the volume of 0.485 N used to titrate the samples

$N$  = the concentration of HCL used (mol/L)

$M(\text{gr})$  = the amount of dry starch acetate sample

Calculation:

- First sample

$$A\% = \frac{(24.1 - 16.6) \times 0.495 \times 43.10^{-3}}{0.5} \\ = 0.31928$$

$$DS = 162 \times \frac{31.928}{300 - 42 \times 31.928} = 1.75$$

- Second sample

$$A\% = \frac{23.9 - 17.4 + 0.495 \times 43.10^{-3}}{0.5}$$

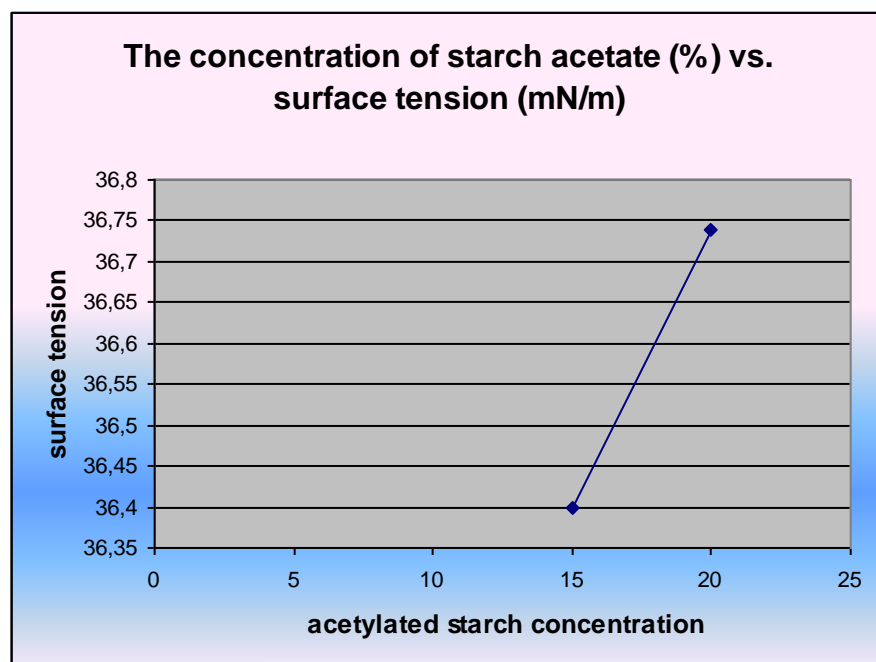
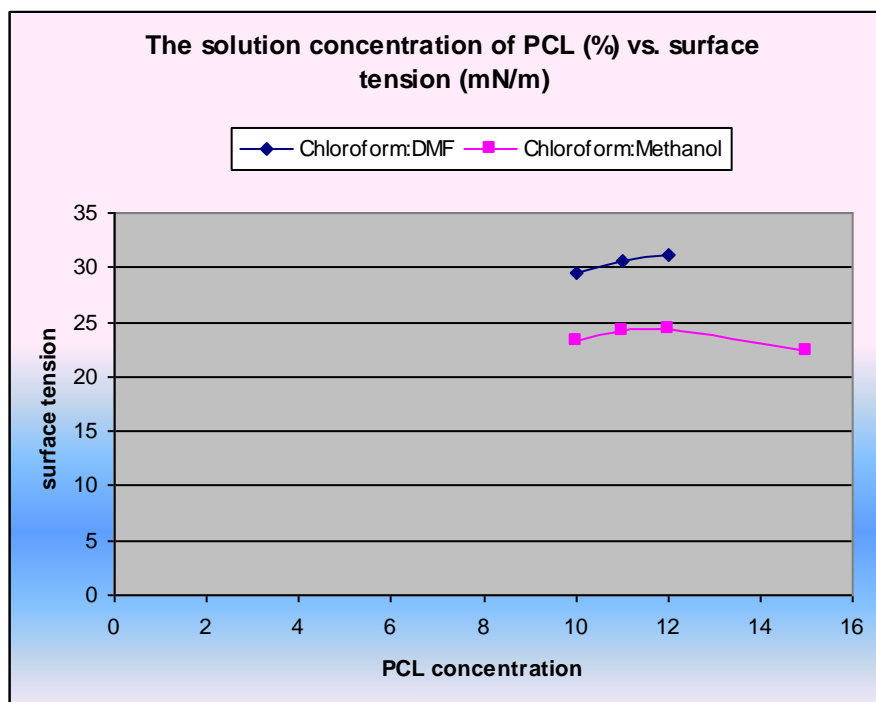
$$= 0.2767$$

$$DS = 162 \times \frac{27.671}{300 - 2 \times 27.671}$$

$$= 1.4$$

The average of DS = 1.6

### C. The graphs of surface tension

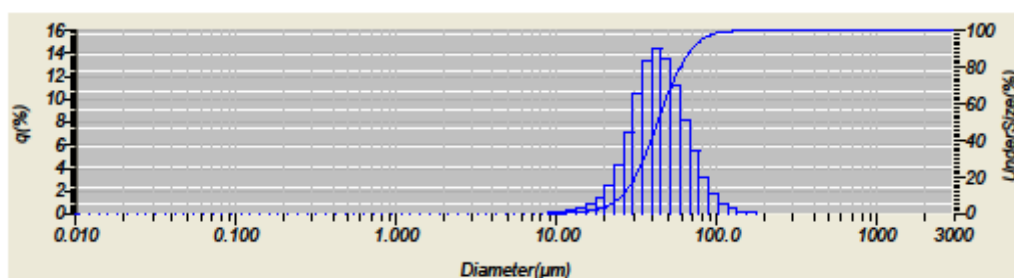


## D. Starch particle size

2009.03.04 16:43:44

# HORIBA Laser Scattering Particle Size Distribution Analyzer LA-950

Sample Name	: starch	Median Size	: 42.78382(μm)
ID#	: 200903041636020	Mean Size	: 46.23560(μm)
Data Name	: 200903041636020	Std.Dev.	: 19.1614(μm)
Transmittance(R)	: 89.5(%)	Geo.Mean Size	: 42.6911(μm)
Transmittance(B)	: 92.9(%)	Geo.Std.Dev.	: 1.4952(μm)
Circulation Speed	: OFF	Mode Size	: 42.0163(μm)
Agitation Speed	: 10	Span	: OFF
Ultra Sonic	: OFF	Diameter on Cumulative %	: (2)10.00 (%) - 26.1320(μm)
Form of Distribution	: Auto		: (9)90.00 (%) - 70.8398(μm)
Distribution Base	: Volume		
Refractive Index (R)	: starch[Nylon( 1.530 - 0.000i),Water( 1.333)]		
Refractive Index (B)	: starch[Nylon( 1.530 - 0.000i),Water( 1.333)]		
Material	: potatoe starch		
Source	: Lachner		
Lot Number	:		
Test or Assay. Number	: 1		



No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)
1	0.011	0.000	0.000	25	0.296	0.000	0.000	49	7.867	0.000	0.000	73	200.000	0.000	100.000
2	0.013	0.000	0.000	26	0.339	0.000	0.000	50	8.816	0.000	0.000	74	229.075	0.000	100.000
3	0.015	0.000	0.000	27	0.389	0.000	0.000	51	10.067	0.129	0.129	75	262.376	0.000	100.000
4	0.017	0.000	0.000	28	0.445	0.000	0.000	52	11.595	0.194	0.323	76	300.518	0.000	100.000
5	0.020	0.000	0.000	29	0.510	0.000	0.000	53	13.246	0.300	0.623	77	344.206	0.000	100.000
6	0.022	0.000	0.000	30	0.584	0.000	0.000	54	15.172	0.480	1.104	78	394.244	0.000	100.000
7	0.026	0.000	0.000	31	0.669	0.000	0.000	55	17.377	0.797	1.901	79	451.586	0.000	100.000
8	0.029	0.000	0.000	32	0.766	0.000	0.000	56	19.904	1.375	3.276	80	517.200	0.000	100.000
9	0.034	0.000	0.000	33	0.877	0.000	0.000	57	22.797	2.429	5.705	81	592.367	0.000	100.000
10	0.039	0.000	0.000	34	1.005	0.000	0.000	58	26.111	4.253	9.959	82	678.504	0.000	100.000
11	0.044	0.000	0.000	35	1.151	0.000	0.000	59	29.907	7.036	16.994	83	777.141	0.000	100.000
12	0.051	0.000	0.000	36	1.318	0.000	0.000	60	34.295	10.464	27.458	84	890.116	0.000	100.000
13	0.058	0.000	0.000	37	1.510	0.000	0.000	61	39.234	13.390	40.826	85	1019.515	0.000	100.000
14	0.067	0.000	0.000	38	1.729	0.000	0.000	62	44.936	14.339	55.165	86	1167.725	0.000	100.000
15	0.076	0.000	0.000	39	1.981	0.000	0.000	63	51.471	13.503	68.668	87	1337.481	0.000	100.000
16	0.087	0.000	0.000	40	2.269	0.000	0.000	64	58.963	11.194	79.862	88	1531.914	0.000	100.000
17	0.100	0.000	0.000	41	2.599	0.000	0.000	65	67.523	8.189	88.051	89	1754.613	0.000	100.000
18	0.115	0.000	0.000	42	2.976	0.000	0.000	66	77.339	5.517	93.568	90	2009.667	0.000	100.000
19	0.131	0.000	0.000	43	3.409	0.000	0.000	67	88.593	3.251	96.819	91	2301.541	0.000	100.000
20	0.150	0.000	0.000	44	3.905	0.000	0.000	68	101.460	1.677	98.497	92	2636.467	0.000	100.000
21	0.172	0.000	0.000	45	4.472	0.000	0.000	69	116.210	0.795	99.292	93	3000.000	0.000	100.000
22	0.197	0.000	0.000	46	5.132	0.000	0.000	70	133.103	0.390	99.682				
23	0.226	0.000	0.000	47	5.897	0.000	0.000	71	152.453	0.206	99.888				
24	0.259	0.000	0.000	48	6.770	0.000	0.000	72	174.616	0.112	100.000				