CHAPTER III
EXPERIMENTAL

3.1 Research Methodology

This research can be divided into three main stages as summarized in Figure 3.1-3.3. The first stage is to investigate the growth of three PEMs that are PDADMAC/PSS, PDADMAC/gelatin and chitosan/gelatin. These PEMs were formed on glass slides, poly (ε-caprolactone) (PCL) films and silicon wafers. The thickness of the coating was determined by an atomic force microscope (AFM) while the hydrophilic/hydrophobic behavior and the stability of the prepared films were confirmed by a contact angle meter and an AFM. The second stage is to study the L929 cell behavior on glass cover slips coated by PEMs. Effects of the outermost layer and the number of layers on cell viability, proliferation and spreading were investigated by the MTT assay and light microscopy. The final stage is to study the L929 cell behavior on PCL nanofibrous scaffolds coated with PEMs. Effects of the top coat on the L929 cell behavior on the coated scaffold were determined.

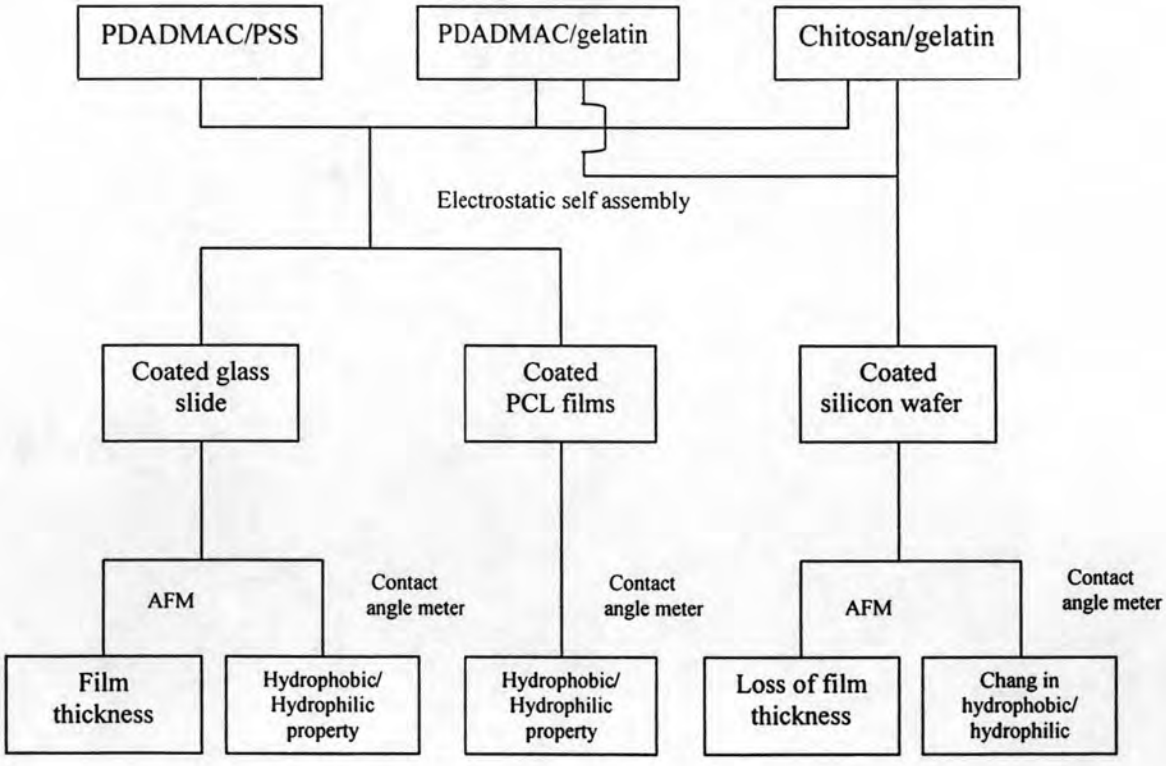


Figure 3.1 Framework of the research (Stage I)

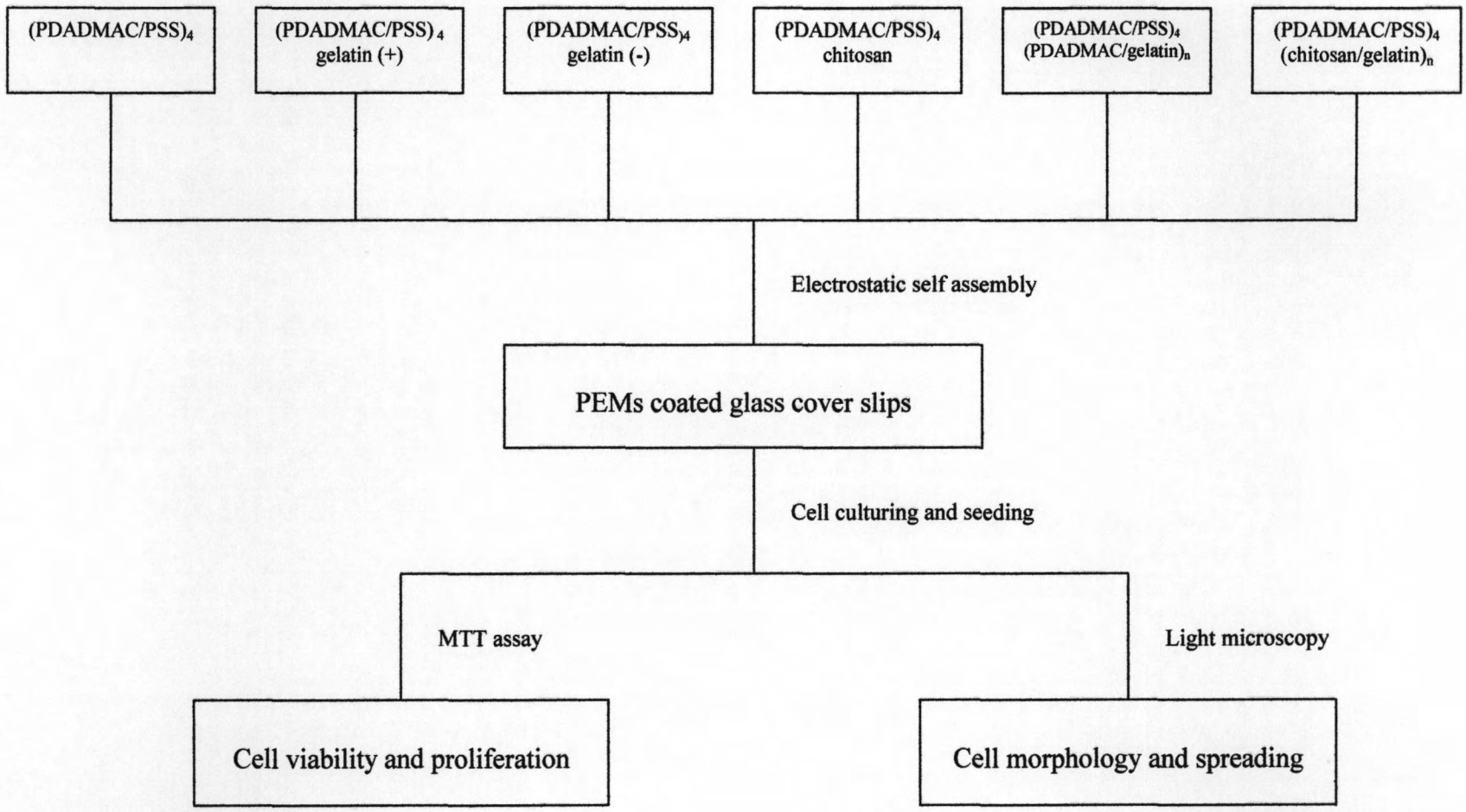


Figure 3.2 Framework of the research (Stage II)

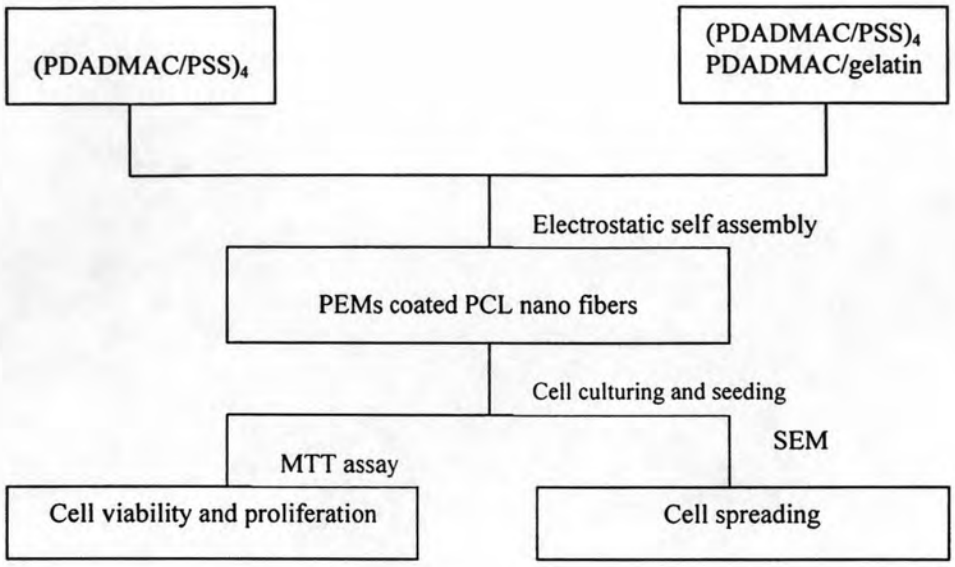


Figure 3.3 Framework of the research (Stage III)

3.2 Materials and Chemicals

3.2.1 Substrates

Substrates used in this research are showed in Figure 3.4.

1. Glass slides (75 x 25 mm. Lab-Scan, Bangkok, Thailand)
2. Silicon wafer (Nove electronic materials, Carrollton, TX)
3. Glass cover slips (No.1 1/2, 22 mm^2, Corning, Midland, MI)

(a) (b) (c)

Figure 3.4 (a) Glass slide (b) Silicon wafer (c) Glass cover slips

3.2.2 The MTT assay kits

The MTT assay kits used in this research are showed in Figure 3.5.

1. 24-well tissue culture plate (Beston, Franklin, NJ)
2. Micropipettes (20-100µl, AS/17-100, Beston, Franklin, NJ)
3. Microlit tips for Micropipettes (AS-TP, Beston, Franklin, NJ)

(a) (b) (c)

Figure 3.5 (a) 24-well tissue culture plate
(b) Micropipettes (c) Microlit tips for Micropipettes

3.2.3 Chemicals

Chemicals used in this research are listed in Table 3.1. All chemicals were used as received without further purification.

Table 3.1 Chemicals used in this research

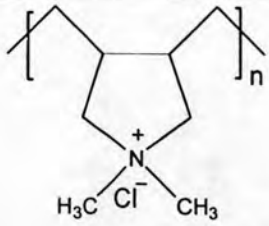
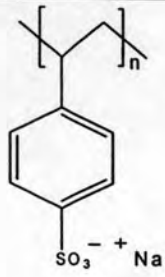
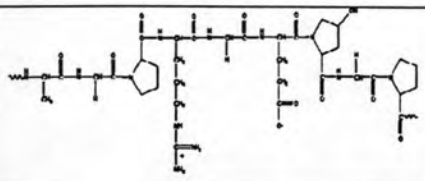
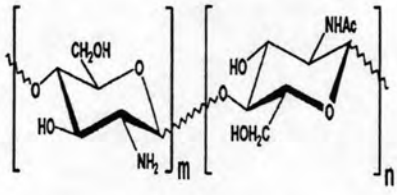
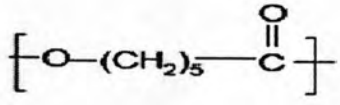
Chemicals	Chemical structure	Properties	Made
Poly (diallydimethyl ammonium chloride) (PDADMACMAC)		- M_w200,000 - 350,000 - bp 100°C - mp -2.8- 0 °C - density 1.04	Sigma St.Louis MO
Poly(sodium 4-styrene sulfonate) (PSS)		- M_w 70,000 - Density0.801 - viscosity 15,000 - 5,000 cps (20% solution, 25 °C)	Sigma St.Louis MO

Table 3.1 (cont.) Chemical used in this research

Chemicals	Chemical structure	Properties	Made
Gelatin		- Type B from bovine skin	Sigma St.Louis MO
Chitosan		- Mw 8 x 10^5 - 84% deacetylation	Fluka Steinheim Germany
Poly (ε-caprolactone)		- Mn 80,000 - mp 60 °C - melt index 1.00 g/10 min	Sigma St.Louis MO
Chloroform	$CHCl_3$	- bp 61.2 °C - Mr 119.38	Lab-Scan Bangkok Thailand
Methanol	CH_3OH	- bp 64.6 °C - Mr 32.042	Lab-Scan Bangkok Thailand
Sodium hydroxide	NaOH	- FW. 40.0 - bp. 1390 °C	Lab-Scan Bangkok Thailand
Sodium chloride	NaCl	- FW. 58443	Lab-Scan Bangkok Thailand
Acetic acid glacial	CH_3COOH	- bp 118 °C	Lab-Scan Bangkok Thailand

3.2.4 Animal tissue cell

L929 mouse fibroblast cells were used for cell culturing and seeding.

3.3 Instruments

Instruments used in this research are listed in Table 3.2

Table 3.2 Instruments used in this research

Instrument	Brand/Model	Made
Automatic dipping machine	Self-made	-
pH/ion meter	Model 25 from Denver Instrument	Qingdao,China
Atomic force microscope	Digital Instrument Nanoscope IIIa	Santa Barbara, CA
Contact angle measurement	Self-made	-
Light microscope	Olympus CK2	Tokyo, Japan
Surface tension	DCAT 11	Filderstadt, Germany
Viscometer	DV-III programmable viscometer	Suarlée(NAMUR), Belgium
High voltage power supply	UCS-30P/CM/VM	Bangkok, Thailand
Spectrophotometer	Genessy 10-S	Rochester, Ny
Scanning electron microscope	JEOL JSM-6400,	Tokyo, Japan

3.4 Experimental procedures

3.4.1 Study of the PEMs formation and their properties

3.4.1.1 Preparation of coated substrates

Glass slides and silicon wafers were cleaned in 70%H_2SO_4 (concentrated) / 30% $H_2O_{2\,(aq)}$ (This solution is called "piranha" which is a strong oxidizer and should not be stored in closed containers) and then in hot 30%H_2O_2/ammonia/water, 1:1:5v/v. Subsequently, the substrates were rinsed in water and dried at ambient temperature. Each substrate was fixed on a stainless steel holder as shown in Figure 3.6 using parafin film.

Figure 3.6 Stainless steel holder

PCL films were prepared by hot pressing at 100°C for 2 min, and then washed with water for 1 min. The PCL films were also fixed on the holders with parafin film.

3.4.1.2 Electrostatic self-assembly of PEMs on Substrate

Multilayer thin films were deposited on substrate using a self-constructed automated dipping machine. Multilayer thin films of cationic and anionic were formed by repeating the following steps on the each substrate; (1) immerse the substrate in positively charged polyelectrolyte solution for 2 min; (2) wash the substrate three times with ultra pure water or buffer solution for 20 sec to remove excess polyelectrolyte (3) immerse the substrate in negatively charged polyelectrolyte for 2 min. (4) remove excess polyelectrolyte solution in the same procedure as in (2). Figure 3.7 shows the Layer-by-Layer deposition technique.

The immersing and washing procedure described above was repeated until the constructed multilayer thin films were obtained.

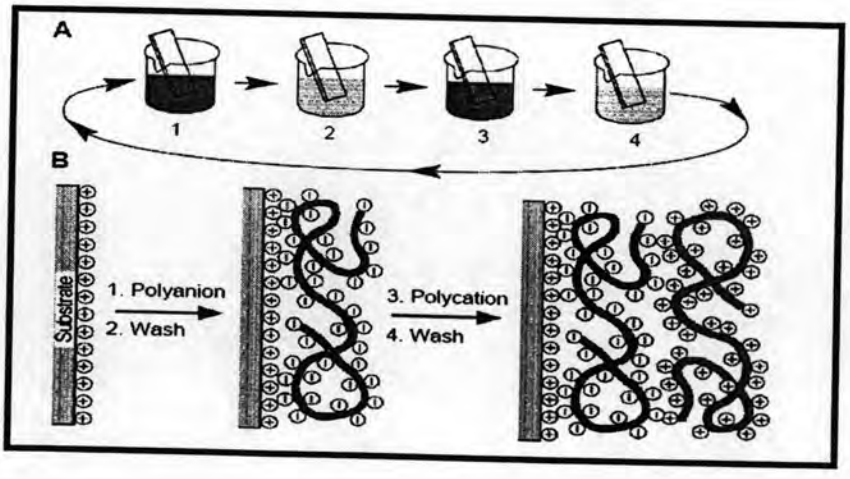


Figure 3.7 Schematic of the layer-by-layer deposition technique

Table 3.3 displays experimental conditions for constructing PEMs on substrates in Part I. PDADMAC and PSS was used without pH adjusting. 0.1 M of NaOH was used to adjust PDADMAC and gelatin solutions to obtain pH of 9. A Chitosan and gelatin solution was adjusted to pH of 6 by using 0.1 M of CH_3COOH and 1 M of NaOH. In both positively charged and negatively charged polyelectrolyte solutions sodium chloride salt was added, and then stirred in order to dissolve. After added the salt, pH of solutions might be adjusted if needed.

Table 3.3 Experimental condition for constructing PEMs on substrates

Code	Concentration (mM)		Salt concentration (M)	Dipping time (min)	pH	Number of layers		
	Positive charge	Negative charge				Glass slides	PCL films	Silicon wafers
PDADMA/ PSS	PDADMAC	PSS	1	2	7	20	4,8,12 16,20	-
	10	10						
PDADM/ gelatin	PDADMAC	Gelatin	0.2	2	9	4,8,12 16,20	4,8,12 16,20	20
	10	10						
chitosan/ gelatin	chitosan	gelatin	0.1	2	6	20	4,8,12 16,20	20
	1	10						

3.4.1.3 Measuring the film thickness

Film thickness of PEMs coated on glass slides was measured by an atomic force microscope (AFM) as shown in Figure 3.8. A scanning probe microscope in tapping mode was operated at ambient temperature. The surface thickness was recorded with a standard silicon tip on a cantilever beam. The spring constant of the cantilever was between 20 and 100 n/m and the length was 125 μm with a resonant frequency of 298 kHz.

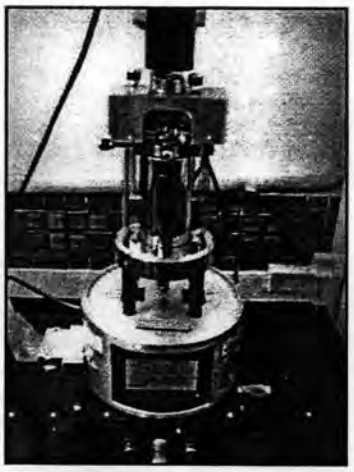


Figure 3.8 Atomic Force microscope, Nano scope IIIa

3.4.1.4 Investigating hydrophilic/hydrophobic property

Contact angles of PEMs coated on PCL films and glass slides were measured in order to investigate the hydrophilic/hydrophobic property. A camera was used to recorded images of the water spreading on the sample. The images were analyzed using computer program. Four different measurements were made for each sample and the average was calculated. All measurements were performed with water pH of 7.4 at ambient temperature.

3.4.1.5 Determining the stability of the PEMs coated in culture medium solution

To study the stability of the PEMs in culture medium solution, PDADMAC/gelatin and chitosan/gelatin PEMs were coated on silicon wafers at 20 layers. Then, the coated substrates were immersed in Dulbecco's minimum Eagle's medium high glucose supplemented with 10% fetal bovine serum for 4, 8, 24 and 48 hr at room temperature. The pH of the cultured medium was maintained at 7.4. The changes in contact angle before and after immersion were determined in order to identify the changes on the hydrophilic/hydrophobic property.

3.4.1.6 Determining loss of surface thickness after exposure to culture medium solution

PEMs made of 20 layers of PDADMAC/gelatin and chitosan/gelatin on silicon wafers were estimated their loss of surface thickness in the term of AFM. After immersed PEMs film in medium solution at 4, 8, 24, and 48hr. The films were

dried in ambient temperature and measure by AFM as the same condition in section 3.4.1.3. The changes in thickness before and after immersion were determined in order to identify the loss of surface thickness.

3.4.2 Study of cell behaviors on PEMs coated glass cover slips

3.4.2.1 Preparation of glass cover slips

Glass cover slips were cleaned using the same method as described in section 3.4.1.1.

3.4.2.2 Electrostatic self-assembly on substrate

Multilayer thin films on glass cover slips were performed by hand dipping. Glass cover slips were immersed in PDADMAC as a positively charged solution for 2 min and then rinsed three times with ultra pure water for 20 sec. These films with positively charged surface were immersed in PSS solution for 2 min. The same rinsing procedures were carried out. Deposition of PDADMAC and PSS layers were accomplished by repeating the same cycle four times. The 8-layer film is labeled as $(PDADMAC/PSS)_4$ which used as the primer layers for adsorbing gelatin and chitosan in the next step.

In order to study the effects of the outermost layer, PEMs coated with $(PDADMAC/PSS)_4$ layer (PSS as the outermost layer) were immersed in a gelatin solution at pH 4 (at this pH gelatin is a positively charge) for 10 min to allow the gelatin to adsorb, Then rinsed three times with buffer of pH 4 for 20 sec and dried at room temperature. The PEMs coated with $(PDADMAC/PSS)_4PDADMAC$ (PDADMAC as the outermost layer) were immersed in gelatin solution at pH 9 (at this pH gelatin is a negatively charge) for 10 min, rinsed with buffer pH 9 and dried respectively. PEMs coated $(PDADMAC/PSS)_4$ which used as the primer layers were immersed in chitosan solution at pH 6 and rinsed using the same procedure. Finally, immersed the primer layer alternately of chitosan and gelatin solution at pH 6 for 10 min each layers and rinsed using the same procedure. The summaries of this step were showed in Table 3.4

Table 3.4 Experimental parameters for constructing PEMs in order to study the effects of the outermost layer on cell behaviors

code	pH	Dipping time for each outermost layers (min)	Types of the outermost layer
$(PDADMAC/PSS)_4$	7	10	primer
$(PDADMAC/PSS)_4$ gelatin (+)	4	10	gelatin (+)
$(PDADMAC/PSS)_4$ gelatin (-)	9	10	gelatin (-)
$(PDADMAC/PSS)_4$chitosan/gelatin	6	10	chitosan/gelatin
$(PDADMAC/PSS)_4$ chitosan	4	10	chitosan

In order to study the effects of number of layers between PDADMAC/gelatin and chitosan/gelatin, PEMs coated with $(PDADMAC/PSS)_4$ were immersed in the solution alternaively between PDADMAC and gelatin at pH 9 for 2 min per each layer. After immersed each solution, rinsed three times with a buffer of pH 9 for 20 sec and dried at room temperature. And PEMs coated with $(PDADMAC/PSS)_4$ were also alternately immersed in solution of chitosan and gelatin at pH 6 for 2 min per each layer, rinsed three times with a buffer pH 6 as the same procedure. All films coated on glass cover slips were dried at room temperature for 24 hours before analysis. The summaries of this step were shown in Table 3.5.

Table 3.5 Experimental parameters for constructing PEM in order to study the effects of number of layers on cell behaviors.

code	pH	Dipping time for each layers (min)	Number of layers
$(PDADMAC/PSS)_4$	7	2	-
$(PDADMAC/PSS)_4$+$(PDADMAC/gelatin)_4$	9	2	8
$(PDADMAC/PSS)_4$ +$(PDADMAC/gelatin)_8$	9	2	16
$(PDADMAC/PSS)_4$+$(chitosan/gelatin)_4$	6	2	8
$(PDADMAC/PSS)_4$+$(chitosan/gelatin)_8$	6	2	16

3.4.2.3 Cell cultured

L929 mouse fibroblast cells were cultured in Dulbecco'modified Eagle's medium high glucose supplemented with 10% fetal bovine serum (FBS). They were incubated at 37^0C in an atmosphere containing 5% CO_2 where the culture medium was changed every 3 days. Cells were released from culture flasks using a trypsin and ethylenediamine tetraacetic (EDTA) Then, the cells were seeded on glass cover slips, plated in 24-well.

3.4.2.4 Cell viability and proliferation test

The MTT assay was used as a measure of relative cell viability according to the methods of Mosmann with minor modifications. Films coated on glass cover slips were sterilized with 70% ethanol solution and rinsed in deionized water for three times before plating with cell. The number of cells determined with nemocytometer. 5×10^4 were cells seeded into the PEMs coated glass cover slips for 4, 8, 24, and 48 hr respectively. Then, MTT solution (5 mg/ml) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well followed by 4, 8, 24, and 48 hr of incubation at 37^0C. Two hundred microliters of DMSO was added to dissolve the fomazan crystals and the solution was mixed using a shaker for 15 min. The absorbance of the solution was measured at 570 nm using a spectrophotometer. Determine the average data from triplicate readings and substract the average value for the blank. For each well, the data were normalized to the initial absorbance of the living cell adhered to the uncoated tissue cultured plate at 0 hr and compared to others PEMs coated. Plot data in the term of percentage of living cells and all data were expressed as mean ± standard deviation.

3.4.2.5 Cell spreading and morphology

L929 cells were plated onto PEMs coated glass cover slips in 24-well plated and growth during 4 – 48 hr to allow cell spreading before imaging. Live cell phase images were obtained using the light microscope equipped (the magnification is 200X.) with a CCD camera and imaging software as shown in Figure 3.9. Staining cells were observed with the microscope. For each PEMs coated, 3 pictures were taken for analyzing cell spreading area and shape factors.

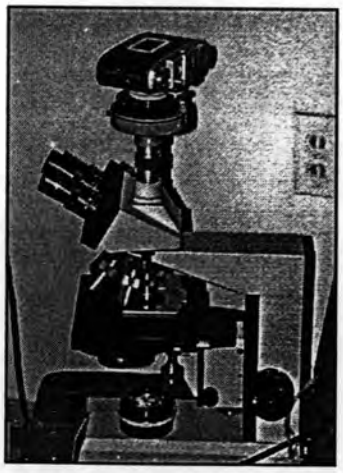


Figure 3.9 Light microscope, Olympus CK2

3.4.3 Study of cell behavior on PEMs coated fibrous scaffolds

3.4.3.1 Preparation of PCL solution

For the electrospinning process, PCL (10.5% w/v) was dissolved in methanol and chloroform (v/v 1:3) for 2 hr with stirring. PCL solution was determined by using a Brookfield viscometer as shown in Figure 3.10. The temperature of the solutions was controlled at 25^0C. The surface tension of the solutions was measured using tension meter as shown in Figure 3.11. The measurement was carries out at 25 ^{0}C and the atmosphere in the measuring chamber was saturated with vapor of the solvent to limit evaporation of the solvent from the pendant drop samples.

Figure 3.10 Brookfield DV-III programmable viscometer

Figure 3.11 Surface tension meter, DCAT 11

3.4.3.2 Preparation of electrospun fibers

The setup of electrospinning apparatus was shown in Figure 3.12. The setup utilized in this study consisted of a plastic syringe and a needle, a ground collection plate, stainless steel sheet on a drum whose rotation speed can be varied, and a high voltage supply. PCL solutions were placed in the syringe fitted with 27 gauge needle. The PCL nanofibers were fabricated by the electrospinning process using the applied voltage of 13 kV. The ground collection plate of aluminum foil was located at a distance of 20 cm from the needle tip. A positive charged jet was formed from the Taylor cone and nanofiber sprayed on to the grounded aluminium foil target. Glass cover slips were placed on the aluminum foil target to collect nanofiber for investigated the cell behaviors. Electrospun fibers were dried at room temperature overnight.

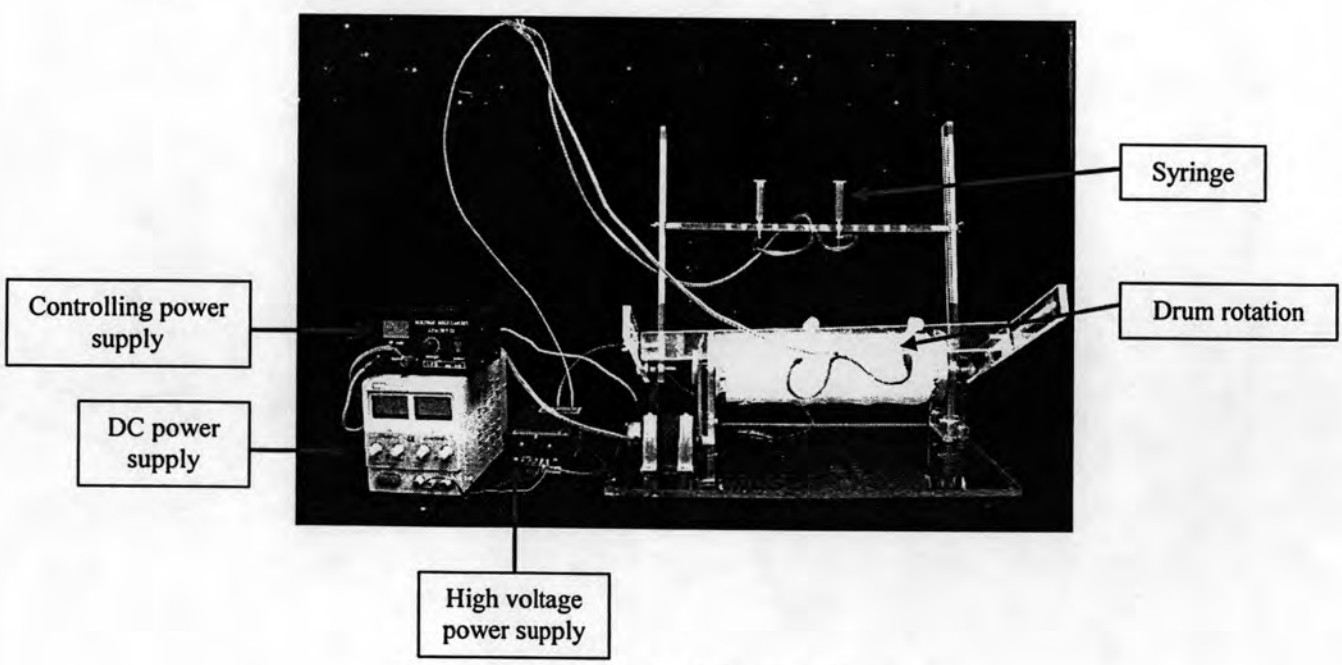


Figure 3.12 The electrospinning set up

3.4.3.3 Electrostatic self-assembly on electrospun nanofibers

In this step, PEMs was constructed by hand dipping from PDAD/PSS and PDADMAC/gelatin. The nanofiber sheets were immersed alternately in polyelectrolyte solutions following the same method as described in section 3.4.2.2. The parameters for constructing PEMs were shown in Table 3.6. The resulting coated and uncoated fiber sheets were tested for cell attachment, proliferation and spreading.

Table 3.6 Experimental parameters for constructing PEM on electrospun fibers

code	pH	Dipping time for each layers (min)	Number of top coated
$(PDADMAC/PSS)_4$	7	2	-
$(PDADMAC/PSS)_4+(PDADMAC/gelatin)_4$	9	2	8

3.4.3.4 Cell culturing and seeding

Before cell culture, the electrospun fibers were placed in 70% ethanol for 10 min to obtain sterility and washed three times in deionized water.

L929 cells were seeded into the coated electrospun fiber scaffolds for 4, 8, 24 and 48 hr, for viability and proliferation analyses. The cell count and evaluation were determined by MTT assay for investigated cell behavior.

3.4.3.5 Cell-scaffold morphology

The morphology of cultured L929 cells was observed by a scanning electron microscope (SEM) as shown in Figure 3.13. The cell-seeded scaffolds were rinsed twice with PBS (pH 7.4), and fixed with 3% glutaraldehyde in 1% calcium chloride for 30 min at 5^0C. After wash in PBS, the cells seeded membrane were dehydrated consecutively in 30%, 50%, 70% , 90% and 100% ethanol for 2 min. Further, substitution to hexamethyl disyloxane was done through the cell seeded membrane. Samples were then critically point dried and covered with a thin layer of gold-palladium through sputtering under an argon atmosphere prior to SEM observation.

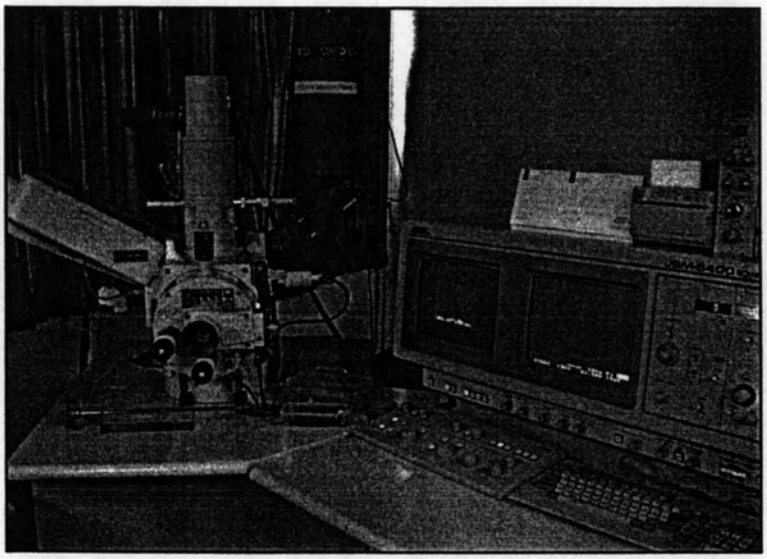


Figure 3.13 Scanning electron microscope, JEOL JSM-6400