

CHAPTER II

MATERIALS AND METHODS

2.1 Colorectal adenocarcinoma cell line (Caco-2 cells) and culture conditions

A Caco-2 cell line is tumorigenic cells derived from human colorectal adenocarcinoma of adult male seventy two years old. Colorectal adenocarcinoma is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract. Colorectal cancers arise from adenomatous polyps in the colon. These mushroom-shaped growths are usually benign, but some develop into cancer over time. Caco-2 cells used in this study were purchased from ATCC. The Caco-2 cells growth properties is adherent to bottom of culture flask and fully differentiate in ten days cultured. Cells were strictly maintained in the cultured following the instruction manual. Briefly, cells (10^5 cells) were routinely cultured as cell adherent manners in Eagle's minimum essential medium (EMEM, Gibco, USA) supplemented with 10% with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (PAA, Austria), placed in 5% $\text{CO}_2(\text{g})$ in humidified air at 37 °C in CO_2 -incubator. The culture medium was changed when the cells were in exponential growth phase (density of cells ~ 70% confluence). The medium was discarded and the cells were washed once with 5 mL sterile phosphate buffer, then added 500 μL of mixture solution of 0.25% (w/v) trypsin and 0.03% (w/v) EDTA into the flask prior to placed the cells at 37 °C for 5 minutes. When cells have detached, added 5 mL completed EMEM. Reduce clumping by forcefully pipetting mixture against side of flask 4-5

times. The suspension of cells was diluted by 2 and 10 time by completed EMEM. The cells obtained from the dilution factor 2 were used for all experiments.

2.2 Determination of Cancer stem cell using antibody to CD34-FITC

CD34, the cell surface molecules will be used for determine the cancer stem cells in caco-2 cell line. The Caco-2 10^6 cells were centrifuged at 7000 rpm for 1 minute and washed once using Phosphate buffer saline (PBS) pH 7.4 at 25°C. For auto fluorescence control drain the supernatant follow add PBS 500 μ L. The analyze sample consecutive addition of anti-CD34⁺ - FITC 10 μ M following by 100 μ L PBS pH 7.4 at 37°C was added into the cell pellets and then incubated at 37°C in the dark.

Ultimately, 400 μ L PBS was added and then the samples were analyzed by flow cytometer.

2.3 Real time Video imaging system for monitoring the behaviors study

The importance tool that we used in this study is the real time imaging system. It is the new in house made system. These imaging monitor tool divided into two parts, first is high resolution microscope Leica DMI 4000B Olympus (magnification 10X-64X) combined with video camera model Super low lux 550TVL Fujiko. This part used to monitor the kinetic behaviors of caco-2 cells in different condition such as classical, 2D, 3D culture system (figure 2.1a). The second part is in house made miniature temperature control device (figure 2.1b). It is very importance for control the cell culture environment. This part used in conjunction with modified medium (CEMI 2009) for maintain the CO₂ pressure levels by compensate the buffering power to stay within pH7 (added 20 mM HEPES 20 mM PBS and 20 mM NaHCO₃⁻). All of

kinetic study the Caco-2 cells behaviors were observed by real time imaging system (Figure 2.1).

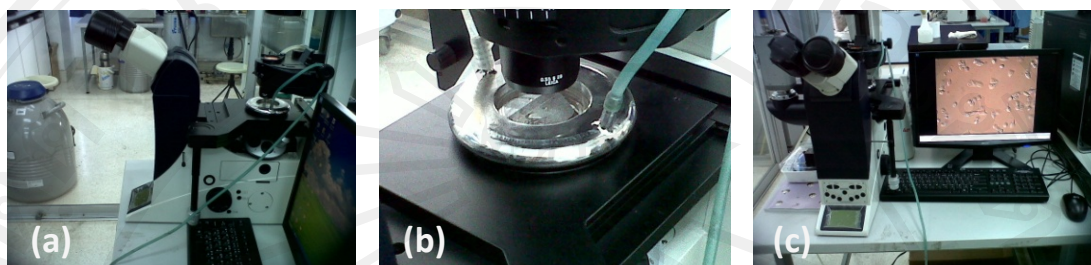


Figure 2.1 The composition of real time video imaging in house tool. (a)The High resolution inverted micro scope Leica DMI 4000B. (b)The temperature control part made from stainless steel. (c) The combine system with CCD camera.

2.4 Video imaging monitor of Caco-2 cell grown on conventional system

The kinetic behavior of Caco-2 cells in conventional system (2D culture system) was observed by Inverted microscope combine with Charge-coupled device (CCD camera) and Super Vertical Helical Scan Video Cassette Recording (S-VHS VCR) for record the video imaging. Prepared Caco-2 cells density 1×10^5 cells/mL final volume 3 mL. Cells cultured on glass Petri dish in EMEM medium. After cells seeded to the glass bottom dish were incubated in temperature controller, an in house made miniature temperature control device at 37°C which is constructed by the laboratory, compensate cabondioxide pressure stable at $5\%\text{CO}_2$ by added 20 mM NaHCO_3^- and 95%humidity. Placed on the sample holder. The Caco-2 cells behaviors were monitor for 3 Days until the differentiation has no observed.

2.5 Drug response pattern monitor in Caco-2 cells

To characterize the small fraction of cancer stem cell in Caco-2 cell line. Drug response pattern of sub population cells was performed. This study observed the different pattern to respond to Doxorubicin, chemotherapy drug used in clinical. Caco-2 cells 10^5 cells/mL final volume 3 mL were seeding on glass bottom dish. Incubated in 5%CO₂ incubator, 95%humidity, 37°C for 24 hour. After that followed by added doxorubicin 100 μM then kinetic was monitored by real time imaging system.

2.6 Caco-2 cells growth pattern on polyacrylamine gel

The new alternative model used in cell culture is 3D model. Many research groups tried to construct the suitable extracellular matrix for cells growth. To mimic the biology and physiology in *in vivo*. The 3D-gel model is a one option to cells culture. In this study used 7% polyacrylamine gel. Caco-2 cells 3×10^6 cells/mL was centrifuge at 7000 rpm for 1 minute. Cells pellet were collected then caco-2 cells were suspended in EMEM medium pH6 containing 7% acrylamide-bis, gentle mixed and then 10 μL ammonium persulfate (10%APS) and 10μL TEMED. The cells mixtures placed on cell culture dish (glass bottom dish). Then the gel phase was form at room temperature where the cells were randomly embedded in side. The fresh EMEM medium was added in order to let the gel immersed into the medium. The culture systems were video imaged as a function of time by real time imaging system. In addition Caco-2 cells cultured in twenty days the morphology can be visualized by staining the cells using a mixture of pirarubicin (THP), acridine orange (AO) and rhodamine B (Rho B). After twenty days the EMEM medium was drained then added

10 μM THP, 5 μM AO and 10 μM Rho B incubate fifteen minutes. Then followed by washing with 3 mL PBS one time. The morphology examines through fluorescence mode of high resolution inverted microscope Leica DMI 4000B.

2.7 Fabrication of 3D-nanofibrous scaffold of 200 μm and 350 μm thickness

The in house made 3D-nanofibrous PVDF membrane was fabricated using electrospinning technique as extensive described by Chanunpanich, Byungsoo et al.2008 (27). Polyvinylidene fluoride the so-called PVDF (Kymar 761, France) of 19 wt% was dissolved in N, N-Dimethylacetamide (DMAA) acetone 4:6 (Aldrich). Syringe having metal needle (1 mm of diameter) was used as the solution reservoir. A drum shaped counter electrode was located opposite to the reservoir. The fibers were collected on the tubular layer at the winding drum of 30 rpm. The distance between the capillary tip and the collector (tip-to-collector distance, TCD) was 10 cm and the applied voltage was 10 kV. A thin polymer fiber is deposited on the collector. The thickness of the fibers was expressed as the spinning time such as 1, 2 and 3 hrs. Electrospun fibers are collected as mix morphology of a non-woven and aligned form. Morphology of the electrospun PVDF nanofiber was observed with scanning electron microscope (SEM) of JEOL model JSM-5410LV. The circular and square 3D-nanofibrous PVDF scaffolds with varied thickness were constructed by fixing the membrane on the structure of polyethylene plastic.

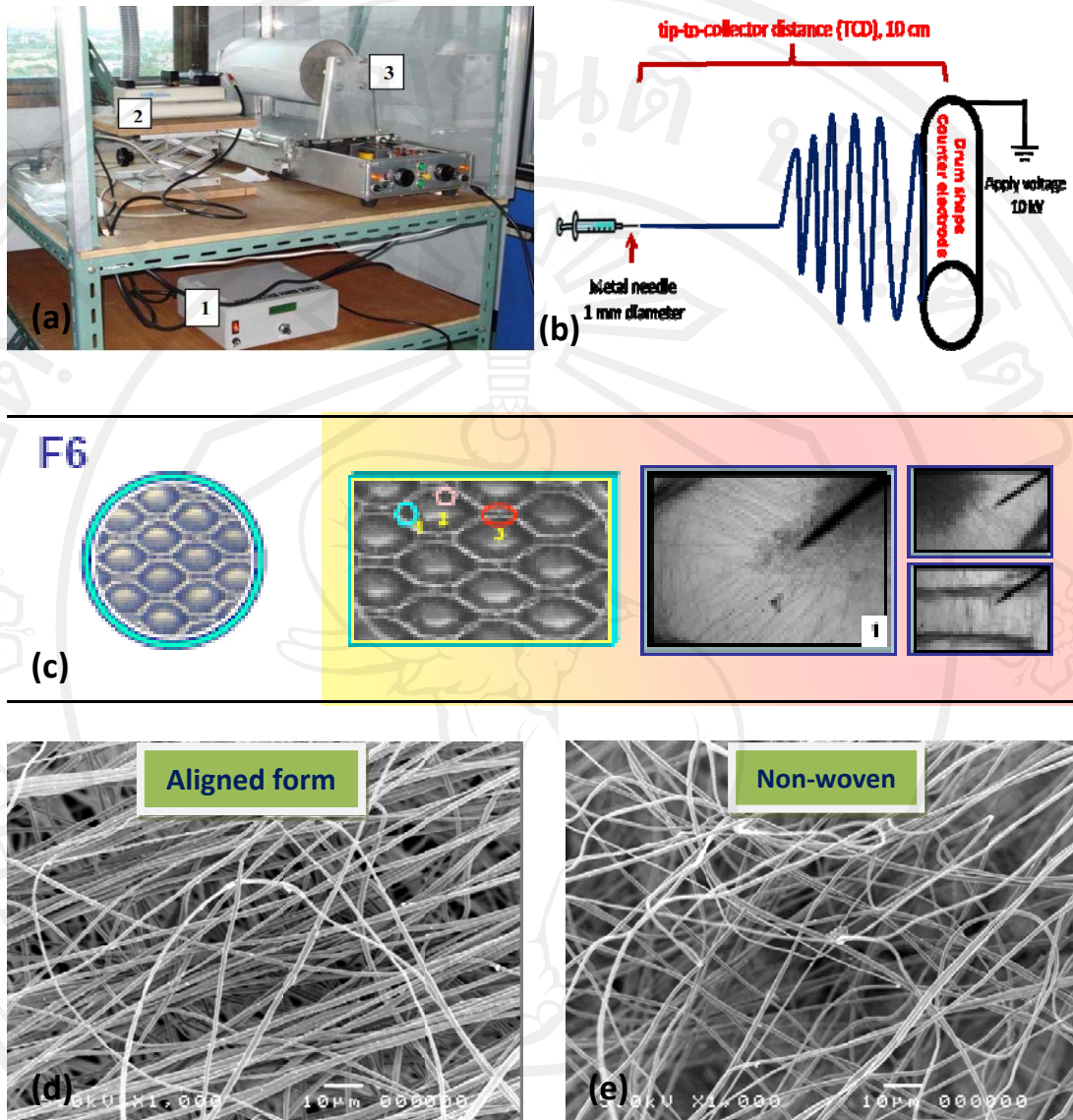


Figure 2.2 (a) The composition of in house made instrument used in scaffold spinning number 1.is applied voltage 10 kV 2.solution reservoirs and 3.is a drum shaped counter electrode. (b) The diagram of electrospinning method (c) The picture shows pattern of fiber in egg-net design. (d), (e) SEM micrograph of nano-fibrous scaffold shows the similar size.

2.8 Video Cells culture and grown on 3D-nanofibrous scaffold

The scaffold, egg-net design (F6FU) as show in figure 2.2 was disinfected by 70% ethanol for 30 minutes then washed three times using sterile Phosphate buffer saline (PBS) pH7.4 at room temperature in biohazard cabinet. Then irradiation by ultraviolet (UVC) for 1 hour finally saturated by EMEM culture medium for 24 hours and incubated at 37 °C for 24 hrs in a CO₂-incubator. Then prepared 50 µL of Caco-2 cells concentration 2×10^6 cells/1sheet of scaffold (surface 0.2515 cm²). Remove culture medium before cells were seeded to the scaffold. After cells seeded, the scaffold was incubated at 37°C, 5% CO₂ and 95% humidity. The 4 mL fresh EMEM medium was added at 4 hours incubation and further incubation for 72 hours. Then the scaffolds were transferred into new 6-well plates and completed with 4 mL of EMEM, incubated at 37 °C in a CO₂-incubator. The culture was maintained by changing the culture medium once a week. The Behavior of Caco-2 cells growth was observed by fluorescence microscope at 4 hours and then every 24 hours.

2.9 Scanning electron microscope and EDX sample preparation

The scaffolds were firstly fixed by immersing 2.5% glutaraldehyde in 0.1M Cacodylate buffer, pH 7.4 for 2 hours at room temperature or at 4° C (in refrigerator) overnight. They were washed 3 times in 0.1 M cacodylate buffer pH 7.4; each 5 minute duration. Then the second fixation was performed by immersing the scaffolds in 1% osmium tetroxide (aqueous) pH 7.4 for 1 hour at room temperature and in a light tight container. The scaffolds were again washed 3 times in 0.1 M cacodylate buffer pH 7.4; each 5 minute duration. The scaffolds were dehydrated by sequentially immersing as follows: 15 min. in 25% ethanol, 15 min. in 50% ethanol, 15 min. in

70% ethanol, 15 min. in 85% ethanol, 15 min. in 95% ethanol and 15 min. in 100% ethanol (EM grade). The scaffolds were then submitted to perform critical point dry which is an automated process takes approximately 40 minutes. The scaffolds were mounted onto metal stub with double sided carbon tape. Finally, a thin layer of gold and palladium were coated over the scaffolds using an automated sputter coater.