CHAPTER III

MATERIALS AND METHODS

3.1 Nanocellulose preparation

Sugarcane bagasse was obtained from Mitr Phol Company in Thailand. The cellulose extraction process involved pretreating the bagasse with a 4% sodium hydroxide solution (1:15 w/v ratio) for 24 hours at 60°C. The pretreated bagasse was then washed with RO water until reaching a neutral pH. To remove color from the sample, a 15% hydrogen peroxide solution (1:10 ratio) was used at 80°C for 2 hours, followed by another RO water wash until a pH of 7 was achieved. The wet cellulose was dried overnight at 50°C in a tray dryer, then mashed and sieved through a 50 µm sieve. A 1% cellulose mixture was prepared by sieving the cellulose with RO water before size reduction.

A hydraulic pilot-scale homogenizer (Microfluidics, M-110EH-30, USA) was shown in figure 3.1 and operated under various pressure conditions (10,000 - 30,000 psi) in combination with an enzymatic process for nanocellulose production. Commercial cocktail enzyme (Novozymes, C-Tech 2, Denmark) was added to the cellulose powder at 1-7% w/w, and the mixture was incubated at 50°C for 96 hours to promote size reduction. Enzymatic hydrolysis was stopped by boiling for 5 minutes. The resulting nanocellulose was then centrifuged at 8,000 rpm for 30 minutes at 25°C and washed three times before being transferred to the mini spray dryer (fig 3.2).

Finally, the nanocellulose was dried using a mini spray dryer (Buchi, B-250, Switzerland) with an inlet temperature of 130°C and the following conditions: 470 L/h air flow rate, 3.5 mL/min feeding rate, 1.4 mm nozzle size, and -50 mbar aspirator pressure drop.

Figure 3.1 Hydraulic pilot-scale homogenizer (Microfluidics, M-110EH-30, USA)

Figure 3.2 Mini spray dryer (Buchi, B-250, Switzerland)

3.2 Production of Nanocellulose based biopolymer scaffold

Design-Expert software version 13 was used to optimize the polymer ratio using the mixture design model. The model operated under the following constraints: (65% ≤ PLA ≤ 90%), (PBS ≤ 35%), (cellulose ≤10%), (PBS + cellulose ≤40%), and (PLA + PBS + cellulose = 100%). The designed experiment was presented in Table 3.1 The responses measured included water contact angle, medium contact angle, and maximum tensile force.

The composite materials were dissolved in N-methyl pyrrolidone (NMP) at 150°C at a total polymer concentration of 30 wt%. Scaffolds were then fabricated using the film

casting technique. Immediately following casting, the scaffolds were immersed in water to remove the solvent. This immersion process was repeated twice. The wet scaffolds were left at room temperature for a few minutes before drying overnight at 50°C. The prepared scaffolds were 8 mm in diameter and were subsequently sterilized using EO (ethylene oxide) gas at the Suranaree University of Technology Hospital.

Table 3.1 The total designed experiment of composited material from Design expert program.

Run	PLA(%)	PBS(%)	nanocellulose (%)
1	77.50	17.50	5.00
2	65.00	26.71	8.29
3	77.50	17.50	5.00
4	65.00	26.71	8.29
5	90.00	7.11	2.89
6	76.30	23.70	0.00
7	90.00	7.11	2.89
8	84.10	11.53	4.37
9	77.50	17.50	5.00
10	65.00	35.00	0.00
11	82.62	7.38	10.00
12	74.69	15.31	10.00
13	82.53	17.47	0.00
14	88.58	1.42	10.00
15	70.14	28.99	0.87
16	77.50	17.50	5.00
17	82.53	17.47	0.00
18	69.24	20.76	10.00
19	88.58	1.42	10.00

3.3 hWJ-MSC isolation and culture

Human umbilical cords (n=2) were obtained from Maharat Nakhon Ratchasima Hospital (Nakhon Ratchasima, Thailand), for which informed consent was obtained from

the mothers in accordance with the Ethics Committee for Research Involving Human Subjects of Suranaree University of Technology (EC-64-125). The cords were approximately 7–10 cm long and were washed with phosphate buffered saline (-) (PBS(-)). hWJ-MSCs were isolated from the umbilical cords and cultured as described previously (Tanthaisong et al., 2017) Briefly, gelatinous Wharton's Jelly tissues were collected and sliced into small pieces (2–5 mm^2), placed in 90-mm × 15-mm culture dishes, and grown in the alpha modification of Eagle's medium (**α**-MEM) enriched with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). The MSCs were expanded until passage 3, cryopreserved with 10% dimethyl sulfoxide (DMSO) in culture media, and then stored in liquid nitrogen.

Human Wharton's Jelly mesenchymal stem cells (hWJ-MSCs) were cultured in collaboration with the Embryo Technology and Stem Cell Research Center, Suranaree University of Technology.

For cell seeding, approximately 1.0 x 10^5 hWJ-MSCs were plated on 4-well dishes (Nunc, Roskilde, Denmark) pre-coated with 0.1% gelatin. The cells were cultured in a complete medium (**α**-MEM supplemented with 10% FBS).

In the following hepatogenic differentiation protocol as shown in figure 3.3., the optimal scaffold was incorporated at the beginning of the process, cells were first cultured in serum-free IMDM (Iscove's Modified Dulbecco's Medium) supplemented with 10 ng/mL bFGF (human basic fibroblast growth factor), 20 ng/mL EGF (epidermal growth factor), 100 µg/mL streptomycin, 100 U/mL penicillin, and 1 mM NaBu (Sodium butyrate) for 3 days (pre-treatment step). Subsequently, the cells were induced to differentiate into the hepatic lineage using serum-free IMDM supplemented with 10 ng/mL bFGF, 40 ng/mL HGF (hepatocyte growth factor), and 5 mM nicotinamide for 7 days (differentiation step). Finally, the cells underwent a maturation step in serum-free IMDM supplemented with 10 ng/mL OSM, 10 nM dexamethasone, and 1% ITS-X for 7 days. The culture medium was changed twice weekly throughout the differentiation process (Panta, et al., 2019).

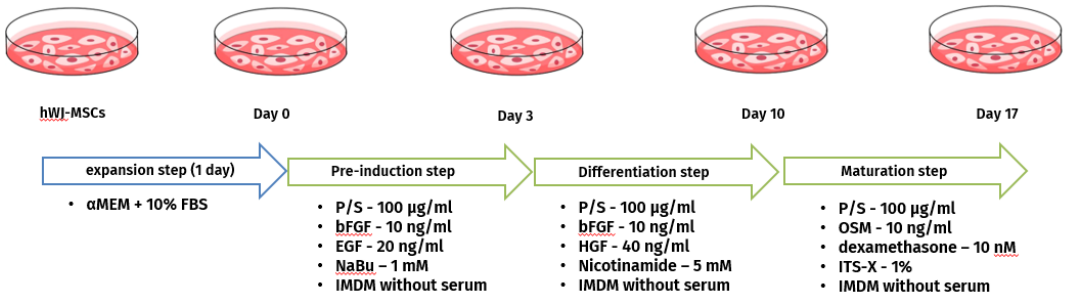

Figure 3.3 Hepatogenic differentiation protocol

3.4 Characterizations

3.4.1 Particle analysis

The average size, zeta potential, and polydispersity index of the nanocellulose samples were characterized using a Zetasizer nano zs instrument (Malvern,UK). The Zetasizer nano zs instrument was shown in figure 3.4.

Figure 3.4 Zetasizer nano zs instrument (Malvern,UK)

3.4.2 Fourier Transform Infrared Spectrometer (FT-IR)

The nanocellulose powder was prepared using a Diamond compression cell (S.T. Japan Europe, Germany) equipped with two disks and a diamond window with a diameter of 3 mm. A thin layer of nanocellulose was added to the window using a needle, followed by compact closure with the two disks.

Synchrotron radiation-based FT-IR spectroscopy was appeared in figure 3.5 and employed to analyze chemical modifications in the nanocellulose. This technique utilizes a synchrotron infrared source at beamline 4.1 of the Thai Synchrotron National Lab, operated in conjunction with a Bruker VEXTEX 70 vacuum FTIR spectroscopy system

linked to a Bruker Hyperion 2000 IR microscope (Bruker Optics, Ettlingen, Germany). The microscope was equipped with a 36X objective and a mercury cadmium telluride (MCT) detector cooled with liquid nitrogen. The nanocellulose sample was measured in transmission mode using a spot size of 20 x 20 μm^2. Background subtraction was performed, and 64 scans were co-added with a spectral resolution of 4 cm^{-1}. Spectral data collected at beamline 4.1 were then analyzed using OPUS 7.5 software.

Finally, principal component analysis (PCA) of the FT-IR spectra was conducted in the wavenumber ranges of 3630-2770 cm^{-1} and 1720-900 cm^{-1} by second derivative analysis. This analysis utilized the Savitzky-Golay smoothing function within the Unscrambler X version 10.4 software.

Figure 3.5 Bruker VEXTEX 70 vacuum FTIR spectroscopy system linked to a Bruker Hyperion 2000 IR microscope (Bruker Optics, Ettlingen, Germany) at beamline 4.1 of the Synchrotron Light Research Institute (Public Organization), Thailand.

3.4.3 Wide-Angle X-ray Scattering (WAXS)

Wide-Angle X-ray Scattering (WAXS) experiments were performed at Beamline 1.3W of the Synchrotron Light Research Institute (Public Organization), Thailand (figure 3.6). The samples were exposed to X-rays with a wavelength of 0.137 nm for 600 seconds at room temperature. The sample-to-detector distance (SDD) was set to 167 mm. The raw data were preprocessed using the SAXSIT program, developed by SLRI staff, to generate the 1D WAXS curve. This curve was then further analyzed using the pseudo-

Voigt function within the SAXSIT software to determine the crystallinity index of the nanocellulose.

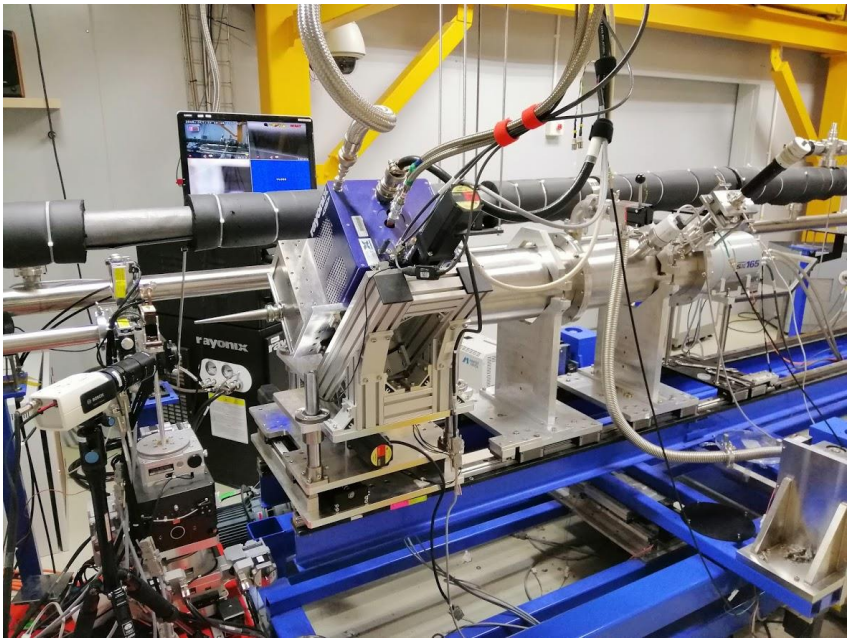

Figure 3.6 Wide-Angle X-ray Scattering (WAXS) instrument at Beamline 1.3W of the Synchrotron Light Research Institute (Public Organization), Thailand.

3.4.4 Thermal characterizations

The thermal stability of the samples was evaluated using thermogravimetric analysis (TGA) under an inert nitrogen atmosphere (flow rate of 30 mL/min) with a TGA instrument (TGA/DSC1, Mettler Toledo, USA) (fig 3.7). Approximately 2 mg of each sample was loaded onto a sample pan and heated from 30 °C to 500 °C at a constant heating rate of 10 °C/min. The onset temperature of weight loss and the temperature corresponding to the maximum degradation rate were determined.

Figure 3.7 TGA instrument (TGA/DSC1, Mettler Toledo, USA)

The thermal properties of the samples were analyzed using differential scanning calorimetry (DSC) (DSC 204F1, NETZSCH, Germany) (fig 3.8) under a nitrogen atmosphere at a heating rate of 10 °C/min. The samples were heated from 20 °C to 250 °C. The glass transition temperature (Tg), melting temperature (Tm), and melting enthalpy (Hm) were determined from the instrument. The degree of crystallinity (X_c) of PLA and PBS in the scaffold was then calculated from the melting enthalpy values according to the following equation:

$$X_c(\%) = \frac{\Delta H_m}{f_p \times \Delta H_m^\circ} \times 100\%$$

where:

- ΔH_m (J/g) is the melting enthalpy of the polymer matrix measured by DSC.
- f_p is the weight fraction of PLA or PBS in the sample.
- ΔH_m° (J/g) is the melting enthalpy of pure crystalline PLA (93 J/g) or PBS (200 J/g) [Hu, & et al., 2018].

Figure 3.8 Differential scanning calorimetry (DSC) (DSC 204F1, NETZSCH, Germany)

3.4.5 Tensile force measurement

Uniaxial tensile testing was performed at ambient temperature to characterize the mechanical properties of the scaffold samples. A TA.XT Plus SMS Stable Micro Systems Texture Analyzer (Stable Microsystems Ltd., Surrey, England) appeared in figure 3.9 and equipped with Texture Expert software was employed for the analysis. The scaffold specimens measured 0.1 mm in width, 50 mm in height, and 20 mm in length. The testing protocol involved subjecting the specimens to a controlled tensile load. The specific parameters for the test were as follows: pre-test speed of 1.0 mm/s, test speed of 1.5 mm/s, post-test speed of 10.0 mm/s, trigger type set to auto-5g, and tare mode disabled.

Figure 3.9 A TA.XT Plus SMS Stable Micro Systems Texture Analyzer (Stable Microsystems Ltd., Surrey, England)

3.4.6 Contact angle measurement

The scaffold was analyzed for the contact angles of ultrapure water and cell culture medium by the sessile drop method in Agriculture and Bioplasma Technology Center, Thailand. Equilibrium contact angles (considered at 60 s) were measured for 5 μL droplet volumes. Determinations were made on 5 different locations for each condition.

3.4.7 Microscopies

The structural surface morphology of the nanocellulose samples and cell adhesion on the scaffold were investigated using field emission scanning electron microscopy (FESEM) (Zeiss, AURIGA, Germany) to assess surface features, FESEM was shown in figure 3.10. Transmission electron microscopy (TEM) (FEI, Tecnai G2 20 TEM, USA) was appeared in figure 3.11 and employed to image the nanocellulose at the microscopy laboratory, Suranaree University of Technology.

Figure 3.10 field emission scanning electron microscopy (FESEM) (Zeiss, AURIGA, Germany)

Figure 3.11 Transmission electron microscopy (TEM) (FEI, Tecnai G2 20 TEM, USA)

3.4.8 Analysis of cytotoxicity

One thousand hWJ-MSCs were replated in 96-well culture plates (SPL Life Sciences, Gyeonggi-do, Korea) and cultured in a culture medium for 24 hours to allow attachment. The cytotoxicity of the optimal scaffold was evaluated by adding it to the culture medium. All cultures were maintained at 37°C for 48 hours in a humidified atmosphere with 5% CO2. The effects of scaffold on cell viability were quantified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The microplate reader (Varioskan LUX, Thermo Scientific, USA) was used to measure absorbance at 540 nm, as shown in Figure 3.12.

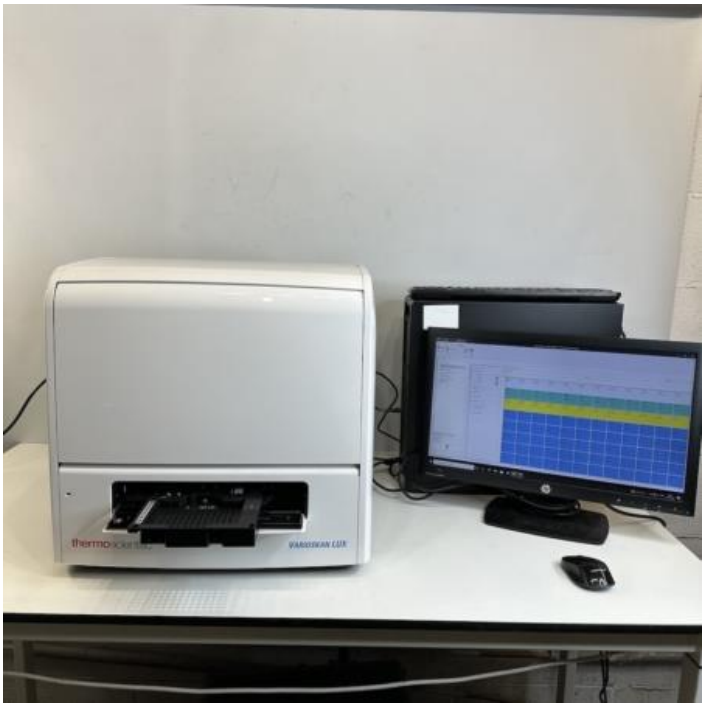

Figure 3.12 microplate reader (Varioskan LUX, Thermo Scientific, USA)

3.4.9 Immunofluorescence staining

Immunofluorescence staining was employed to evaluate protein expression profiles in cells undergoing a 17-day differentiation process on the scaffold. Cells were harvested on day 17 and fixed with 4% paraformaldehyde (PFA) for 15 minutes. Subsequently, a blocking and permeabilization step was performed using a solution containing BSA, normal goat serum, sodium azide, and Triton-X 100 for 2 hours at 37°C. The cells were then incubated overnight at 4°C with a panel of primary antibodies targeting specific proteins of interest, including alpha-fetoprotein (AFP), cytokeratin 18 (CK18), and albumin (ALB). Following incubation, the cells were washed and subsequently exposed to corresponding secondary antibodies for 2 hours. Nuclei were visualized using DAPI staining, and protein expression was observed using a Confocal Laser Scanning Microscope (CLSM-Upright Ni-E, Nikon, Japan), which instrument appeared in figure 3.13.

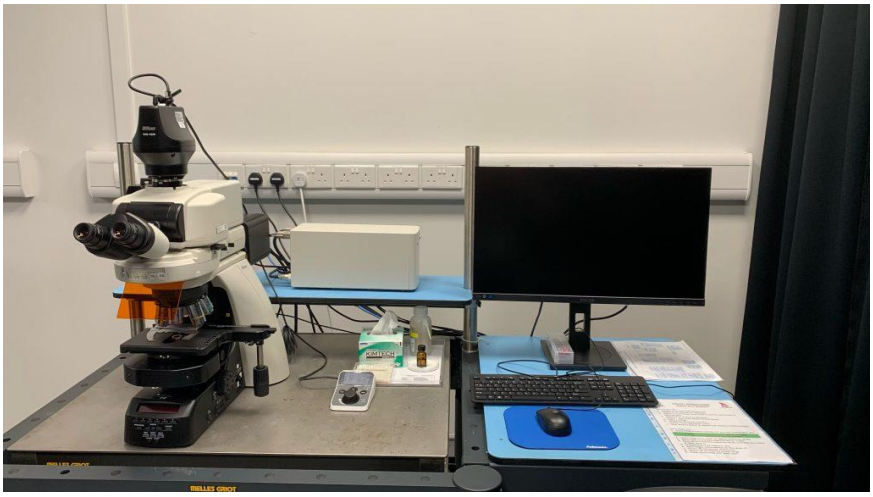

Figure 3.13 Confocal Laser Scanning Microscope (CLSM-Upright Ni-E, Nikon, Japan)