CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

N-hydroxysuccinimide (NHS, 98%), triethylamine (TEA, 99.5%), pyrene (98%), 2-nitrobenzyl bromide (98%), *N,N'*-dimethylethylenediamine (85%), 4-cyanopentanoic acid dithiobenzoate (CPADB, 97%), 4,4'-azobis(4-cyanovaleric acid) (ACVA, 75%), and albumin-fluorescein isothiocyanate conjugate (BSA-FITC) were purchased from Sigma-Aldrich and used as received. *N*-isopropylacrylamide (NIPAAm, 97%) was obtained from Sigma-Aldrich and recrystallized twice in hexane before use. Acrylic acid (AA, 99%) was obtained from Sigma-Aldrich and purified by distillation under reduced pressure prior to use. Pentafluorophenol (99.0%) was commercially available from Merck and used as received. Azobisisobutyronitrile (AIBN, 98%) and dicyclohexylcarbodiimide (DCC, 99%) were obtained from Fluka and used as received. Acryloyl chloride was purchased from Acros and used as received. H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS) was purchased from GenScript (USA). Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from InVitromex. Fibroblast (L929) cell line was obtained from ATCC.

3.1.2 Solvents

All solvents used for reactions are reagent grade and used as received, otherwise specified. Anhydrous *N,N'*-dimethylformamide (DMF, 98.8%), anhydrous dimethylsulfoxide (DMSO, 99%), and anhydrous tetrahydrofuran (THF, 99.9%) were obtained from Sigma-Aldrich. Anhydrous 1,4-dioxane (99.9%) were obtained from Merck. Dichloromethane was dried over CaH_2 under reflux and nitrogen atmosphere. Phosphate buffered saline (PBS) pH 7.4 (10 mM PBS consisting of 10 mM phosphate buffer, 137 mM NaCl, and 2.7 mM KCl) was purchased from Sigma-Aldrich. Solutions were made with MilliQ water purified by Millipore Milli-Q system that involves reverse osmosis, ion exchange, and filtration steps (18.2 MΩ). The NMR solvents such

as $CDCl_3$ (99.8% D), DMSO-d_6 (99.9%), and D_2O (99.9% D) were obtained from Cambridge Isotope Laboratories, Inc. (USA).

3.2 Instrumentations

3.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^1H NMR spectra were recorded on a Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz, and ^{19}F NMR spectra were recorded on a Bruker DRX 400 FT-NMR spectrometer. Chemical shifts (δ) were reported in part per million (ppm) relative to tetramethylsilane (TMS) signal as a reference.

3.2.2 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra were recorded with a Nicolet Impact 6700 FT-IR spectrometer with 32 scans at a resolution of 4 cm^{-1} in a frequency range of 400-4000 cm^{-1}. Samples were pressed into potassium bromide (KBr) pellets.

3.2.3 Gel Permeation Chromatography (GPC)

Molecular weight and molecular weight distributions (M_w/M_n) of synthesized polymers were analyzed by GPC using Waters 600 controller chromatograph equipped with HR1 and HR4 columns (Waters, M_W resolving range = 100-500,000 g/mol) at 35°C and refractive index (RI) detector (Waters 2414). THF was used as an eluent with the flow rate of 1.0 mL/min. Sample injection volume was 80 µL. Five polystyrene standards (996-188,000 g/mol) were used for generating a calibration curve.

3.2.4 Dynamic Light Scattering (DLS)

Average sizes of micelles in hydrodynamic diameter were measured using a DLS instrument (Zetasizer Nano ZS, Malvern Instrument Ltd., U.K. equipped with a He-Ne laser beam at 658 nm) at a fixed scattering angle of 173°. The sample refractive index (RI) was set at 1.59 for polystyrene. The dispersant viscosity and RI were set to 0.89 $Ns \cdot m^2$ and 1.33, respectively. All samples (1.0 mg/mL) were filtered through a 0.45 µm syringe filter prior to analysis, and each measurement was

repeated at least ten times to obtain the average value with an equilibrium time of 5 min before each measurement.

3.2.5 Scanning Electron Microscopy (SEM)

The surface morphology of electrospun fibers was observed using a JEOL Model JSM-6610/LV scanning electron microscope (USA) and analyzed with an accelerating voltage of 15 kV. A small piece of electrospun fibers on aluminum foil was cut and placed on SEM stub using a double-sided adhesive carbon tape. Sample was sputter-coated with gold prior to imaging. The fiber diameters were measured by using a SemAfore 5.21 for 50 fibers per sample.

3.2.6 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) studies were performed on a JEOL Model JEM-2100 electron microscope operating at an acceleration voltage of 100 kV. Samples were prepared by drop-casting of a given micelle solution onto carbon-coated copper grid and excess solution was carefully blotted off using filter paper. The samples were then air-dried at room temperature before measurement.

3.3 Experimental Procedure

3.3.1 Synthesis of *ortho*-nitrobenzyl (ONB) protected diamine

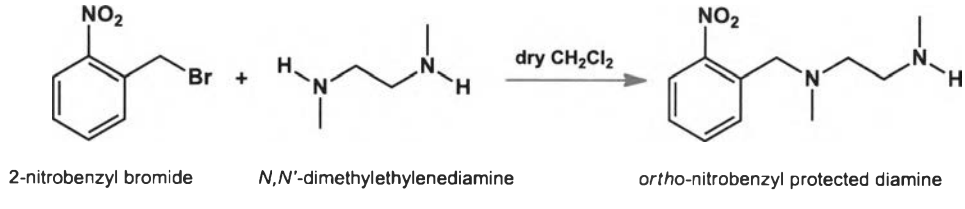

Figure 3.1. Synthesis of mono ONB-protected diamine.

ONB-protected diamine was synthesized according to a method modified from that of Zhao, *et al.*[26]. 2-Nitrobenzyl bromide (1.0 g, 4.6 mmol), *N,N'*-dimethylethylenediamine (4 mL, 37.2 mmol), and dry dichloromethane (20 mL) were added into a round bottom flask. The reaction mixture was stirred in the dark place

and at room temperature overnight. After that, the solution was extracted with distilled water for 3 times. The filtrate was dried over anhydrous sodium sulfate (Na_2SO_4) and dichloromethane was then removed using rotary evaporator. The pure yellow liquid was obtained (0.69 g (3.2 mmol, 70% yield). The synthetic strategy of ONB-protected diamine is shown in Figure 3.1.

^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm: 7.89-7.272 (m, 4H), 3.79 (d, 2H), 2.63-2.52 (m, 4H), 2.46 (s, 3H), 2.04 (s, 3H).

3.3.2 Synthesis of monomers

3.3.2.1 Synthesis of *N*-acryloxysuccinimide (NAS)

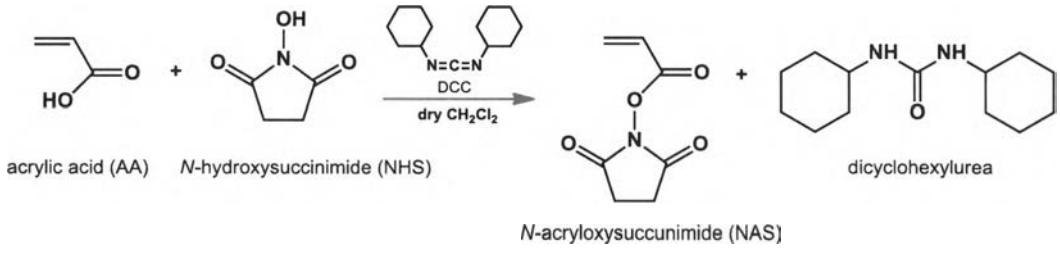

Figure 3.2. Synthesis of *N*-acryloxysuccunimide (NAS).

N-acryloxysuccunimide (NAS) monomer was synthesized by a coupling reaction of acrylic acid (AA) with *N*-hydroxysuccinimide (NHS) (Figure 3.2) according to the previously reported procedure [27]. AA (1.37 mL, 0.02 mol) was added dropwise to a stirred solution of NHS (2.30 g, 0.02 mol) in 20 mL of dry dichloromethane on ice bath. *N,N'*-dicyclohexylcarbodiimide (DCC) (4.12 g, 0.02 mol) in 10 mL of anhydrous dichloromethane was slowly added to the mixture solution under stirring. The solution was stirred at room temperature for 48 h. The insoluble dicyclohexylurea was removed by filtration. After evaporation of solvent, the crude product was purified by column chromatography (column material: silica gel) with hexane:ethyl acetate as eluent. The pure white solid crystal of NAS was obtained (1.93 g, 0.011 mol, 57% yield) and stored in refrigerator at -20°C.

^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm: 2.85 (s, 4H), 6.0-7.0 ppm (m, 3H);

FT-IR (KBr mode): 1810, 1781 cm^{-1} (succinimidyl groups), 1737 cm^{-1} (C=O reactive ester band)

3.3.2.2 Synthesis of pentafluorophenylacrylate (PFPA)

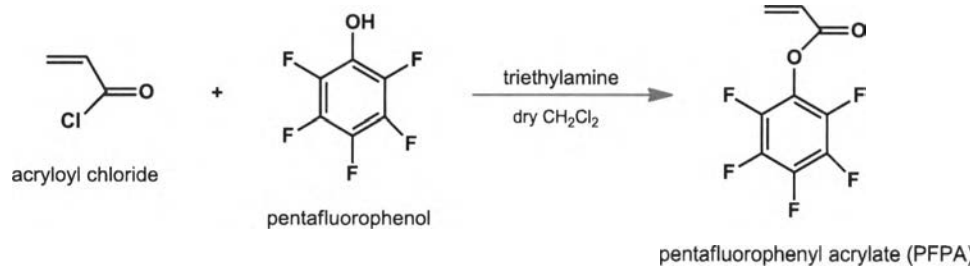

Figure 3.3. Synthesis of pentafluorophenylacrylate (PFPA).

Pentafluorophenyl acrylate (PFPA) was synthesized according to the method of Jochum, *et al.*[28]. Pentafluorophenol (40.0 g, 0.22 mol) was dissolved in 250 mL of dry dichloromethane in round bottom flask. Triethylamine (TEA) (36.2 mL, 0.26 mol) was slowly added to the reaction mixture on ice bath, and acryloyl chloride (21.1 mL, 0.26 mol) was then slowly added. After being removed from the ice bath, the reaction mixture was stirred at room temperature overnight. The precipitate of triethylamine hydrochloride salt was filtered, washed thoroughly with dichloromethane. The filtrate was extracted twice with acidic water (pH = 2.0), and twice with basic solution (Na_2CO_3), and finally twice with DI water. The organic phase was dried over anhydrous sodium sulfate (Na_2SO_4), and dichloromethane was removed using rotary evaporator. The crude product was further purified by column chromatography (column material: silica gel; solvent: petroleum ether). The pure colorless liquid was obtained (26.3 g, 0.16 mol, 75% yield) and stored in refrigerator at -20°C. The synthetic strategy of PFPA is shown in Figure 3.3.

^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm: 6.70 (d, 1H), 6.35 (dd, 1H), 6.16 (d, 1H);

^{19}F NMR ($CDCl_3$): δ/ppm: -162.77 (d, 2F), -158.39 (t, 1F), -153.02 (d, 2F);

FT-IR (ATR-mode): 1772 cm^{-1} (C=O reactive ester band), 1516 cm^{-1} (C=C aromatic band)

3.3.3 Synthesis of homopolymer and copolymers by RAFT polymerization

3.3.3.1 Synthesis of poly(NIPAAm-*ran*-NAS) (P(NIPAAm-*r*-NAS))

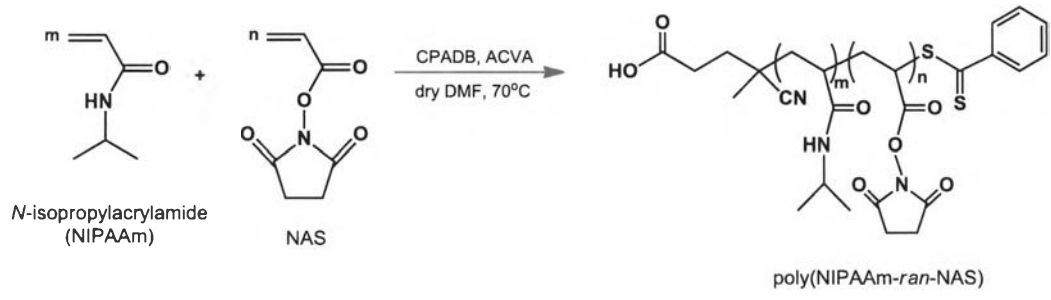

Figure 3.4. Synthesis of poly(NIPAAm-*ran*-NAS).

N-isopropylacrylamide (NIPAAm) (1.91 g, 16.9 mmol), NAS (0.32 g, 1.89 mmol), 4-cyanopentanoic acid dithiobenzoate (CPADB) (13 mg, 0.047 mmol), and 4,4'-azobis (4-cyanovaleric acid) (ACVA) (3.28 mg, 0.012 mmol), were dissolved in 5 mL of dry DMF. The vial was sealed with a rubber septum and the solution was purged with nitrogen gas for 30 min to remove dissolved oxygen. The vial was then immersed into a thermo-stated oil bath at 70°C for a set of reaction time. The samples were withdrawn from the polymer solution every 2 h and then stopped by quenching the reaction in an ice bath. The monomer conversion, average molecular weight, and molecular weight distribution (PDI) of crude samples were monitored by ^{1}H NMR and GPC, respectively. The remaining polymer solution was purified by dialysis against DMSO for 2 days and in DI water for 3 days and then lyophilized to yield pink powder. In order to study the comonomer composition and molecular weight of the copolymer, the mole ratio of NIPAAm to NAS monomers in feed and the ratio of monomer to CTA were varied, respectively (Figure 3.4).

3.3.3.2 Synthesis of poly(pentafluorophenyl acrylate) (PPFPA)

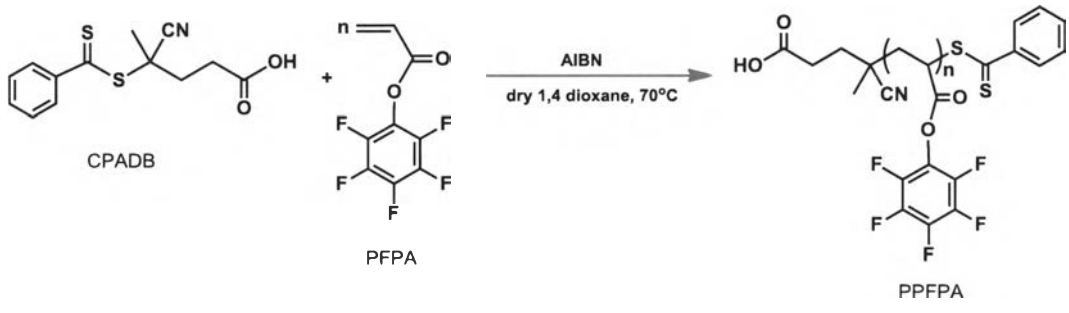

Figure 3. 5. Synthesis of poly(pentafluorophenyl acrylate) (PPFPA).

PPFPA was synthesized according to the method of Jochum, *et al.*[28]. Briefly, PFPA monomer (4.80 g, 20 mmol), CPADB (11 mg, 0.04 mmol), and AIBN (0.65 mg, 0.004 mmol) were added to a vial followed by 10 mL of dry 1,4-dioxane. The vial was sealed with a rubber septum and the solution was purged with argon gas for 30 min. The vial was then immersed into a thermo-stated oil bath at 70°C for 2 h. After immersing for 2 h, the polymer solution was precipitated in methanol, centrifuged, and dried under vacuum at room temperature. The dried polymer was then dissolved in THF and precipitated again in methanol. This procedure was repeated two times. The resulting PPFPA homopolymer was subsequently used as a macro chain transfer agent (macro CTA) for the polymerization of NIPAAm. The synthetic strategy of PPFPA is shown in Figure 3.5.

3.3.3.3 Synthesis of poly(PFPA)-*block*-poly(NIPAAm) (PPFPA-*b*-PNIPAAm)

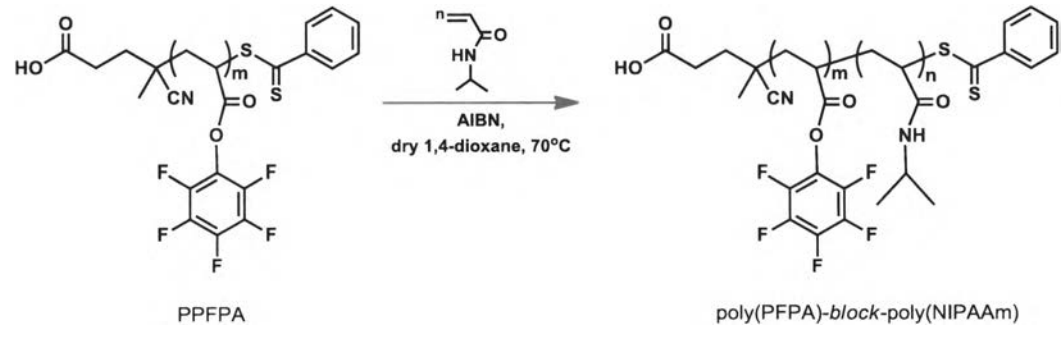

Figure 3.6. Synthesis of poly(PFPA)-*block*-poly(NIPAAm) (PPFPA-*b*-PNIPAAm).

For the synthesis of diblock PPFPA-*b*-PNIPAAm copolymers using RAFT polymerization, it is advisable to start with the monomer that shows higher transfer ability for the selected CTA. In this case, PPFPA was first synthesized and used as a macro CTA for PNIPAAm synthesis [29]. The purified PPFPA (0.32 g, 0.05 mmol), NIPAAm (0.57 g, 5.04 mmol), and AIBN (0.5 mg, 0.003 mmol) were added to a vial followed by 6 mL of dry 1,4-dioxane. The vial was sealed with a rubber septum and the solution was purged with nitrogen gas for 30 min. Polymerization was conducted under nitrogen atmosphere at 70°C for 12 h. The polymer solution was precipitated in hexane, centrifuged, and finally dried under vacuum at room temperature. The dried copolymer was then dissolved in THF and precipitated again in hexane. This procedure was repeated two times. ^{1}H NMR and GPC analysis were used to confirm the formation of the block copolymers. The synthetic strategy of PPFPA-*b*-PNIPAAm is shown in Figure 3.6.

3.3.3.4 Synthesis of poly(NIPAAm-*ran*-PFPA) (P(NIPAAm-*r*-PFPA))

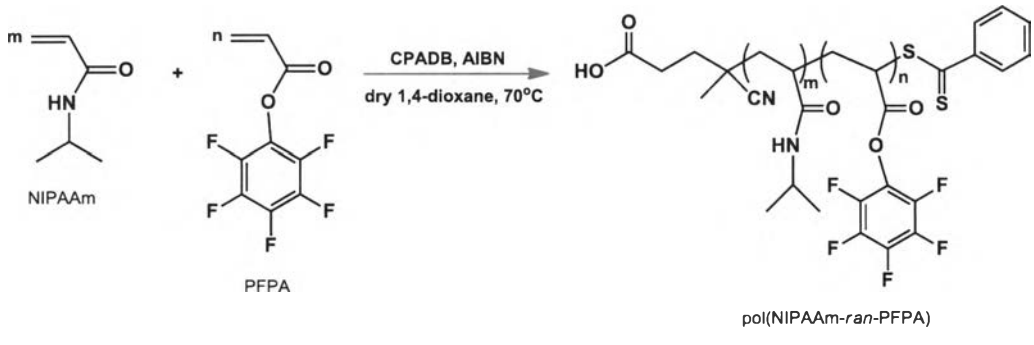

Figure 3.7. Synthesis of poly(NIPAAm-*ran*-PFPA) (P(NIPAAm-*r*-PFPA)).

NIPAAm (1.13 g, 10 mmol), PFPA (2.38 g, 10 mmol), CPADB (11 mg, 0.04 mmol), and AIBN (0.8 mg, 0.005 mmol), were dissloved in 10 mL of dry 1,4-dioxane. The synthetic and the purification method are the same as those of PPFPA-*b*-PNIPAAm. The synthetic strategy of P(PFPA-*r*-NIPAAm) is shown in Figure 3.7.

3.3.3.5 Post-polymerization modification of activated ester copolymers with ONB-protected diamine

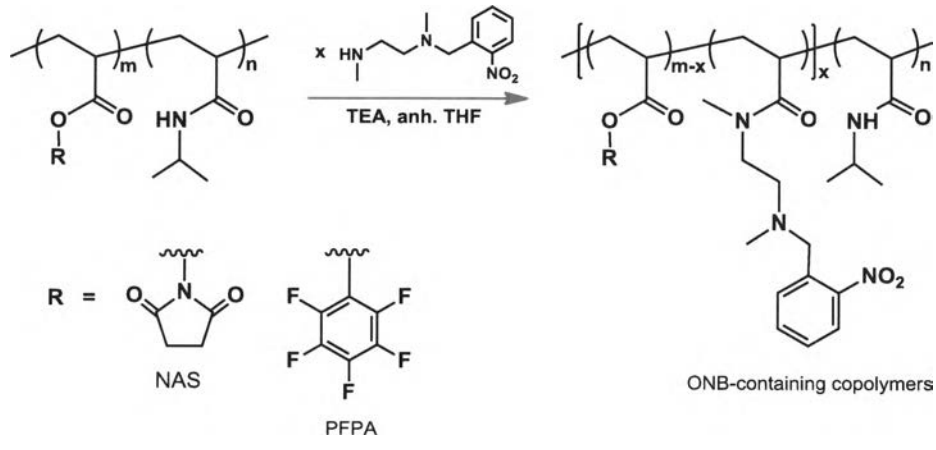

Figure 3.8. Schematic representation of the nucleophilic substitution of activated ester copolymers with ONB-protected diamine.

Herein, light responsive moieties of ONB containing random and block copolymers of P(NIPAAm-*r*-NAS), P(NIPAAm-*r*-PFPA), and PPFPA-*b*-PNIPAAm were prepared for the first time via post functionalization of PNAS or PPFPA part as shown in Figure 3.8. The copolymers (0.5 g, 1 equiv. of activated ester) were dissolved in 5 mL of dry THF or DMF under nitrogen atmosphere for 30 min. Separately the TEA (0.1 equiv), ONB-protected amine (0.5 equiv.) was dissolved in 1 mL of dry THF. The ONB-protected amine solution was quickly added to the polymer solution and continued purging with nitrogen gas for 20 min. The solution was stirred at room temperature for 24 h. The resulting copolymer was purified by precipitation in hexane, centrifuged, and vacuum dried at room temperature overnight.

3.3.4 Preparation of micelles

Membrane-dialyzed method [6] was used to prepare micelles through self-assembly of PPFPA-*b*-PNIPAAm and ONB containing PPFPA-*b*-PNIPAAm block copolymers. The block copolymers (10 mg) were dissolved in 5 mL of THF. The solution were put into a dialysis tube (molecular weight cutoff = 3,500 g/mol) and

dialyzed against water at 3-4°C for 24 h. The aqueous solution in the dialysis tube was then lyophilized to leave a powder of dry micelles.

3.3.5 Electrospinning

P(NIPAAm-*r*-NAS), P(NIPAAm-*r*-PFPA), and ONB containing copolymers were fabricated as fibers by electrospinning. The electrospinning apparatus consisted of a variable syringe pump (ProSense B.V. Laboratory & process equipment model NE1000, Netherlands) and a high voltage power supply (Gamma High Voltage Research, model ES30P, USA). The copolymer with different concentrations (10-30% w/v) were dissolved in acetone and in THF/DMF (3:1, v/v), respectively. The polymer solutions were then stirred at room temperature for 24 h. The polymer solution was loaded in a 5 mL plastic syringe equipped with a metallic needle. The syringe was fixed horizontally on the syringe pump and an electrode of high power supply was clamped to the metal needle tip. A grounded stationary rectangular metal collector covered with a piece of clean aluminum foil was used for the fiber collection. The distance between the needle tip and the collector was set to 20 cm. The flow rate of polymer solution was fixed at 3 mL/h, and the applied voltage was set at 20 kV. The electrospun fibers were collected on a flat aluminum foil and then vacuum dried at room temperature overnight prior to further studies.

3.3.6 Immobilization of GRGDS peptide on electrospun fibers

GRGDS peptide 0.05 M was dissolved in PBS buffer pH 7.4. The electrospun fibers were cut into small circle pieces (15 x 15 mm) and placed into the bottom of 24-well plates. The GRGDS peptide solution was pipetted into each well of the electrospun fibers. After reacting for 24 h, the samples were rinsed twice with PBS and DI water, respectively. The copolymer fibers were then dried under vacuum overnight.

3.3.7 Cell viability using MTT Assay

Fibroblast (L929) cell line was used to study the cell adhesion and proliferation for electrospun fibers. The L929 cells were cultured in RPMI 1640 medium

supplemented with 5% fetal bovine serum (FBS), penicillin (100,000 U/L) and streptomycin (100 mg/mL). They were incubated at 37°C in atmosphere containing 5% CO_2 where the culture medium was changed every 3 days.

For cell culture, the electrospun samples were sterilized with UV light overnight. The samples were then transferred to the bottom of 24-well tissue culture polystyrene (TCPS) plate. Three replicated samples were used for each condition. Approximately $2x10^4$ of L929 cells in 0.2 mL culture medium were pipetted into each well containing the substrates as well as into the bottom of TCPS plates as a control and then incubated under 5% CO_2 at 37°C.

MTT assay was used to investigate cell adhesion and proliferation. After 6 h of incubation, the culture medium was removed to discard the unattached cell and the 0.2 mL fresh culture medium was pipetted into each well followed by 10 µL of 0.5 mg/mL MTT/normal saline solution. After incubation for 4 h, the supernatant solution was removed and 150 µL of DMSO was pipetted into each well to dissolve the purple crystals of formazan. Next, 25 µL of 0.1 M glycine (pH 10.5) was added. The optical density of sample was measured using a microplate reader at the wavelength at 540 nm. Cell adhesion ratio on each surface was evaluated using Eq. (3.1) where $OD_{sample,12h}$ represents the optical density on the different polymeric surfaces and $OD_{TCPS,12h}$ represents the optical density on TCPS surfaces, which was utilized as a standard.

$$\text{Cell adhesion ratio (\%)} = \frac{OD_{sample,6\ h}}{OD_{TCPS,6\ h}} \times 100 \qquad (3.1)$$

The measurement of changes in number of proliferated cells on substrates was determined at the incubation time point of 1, 3 and 5 days. The cell proliferation ratio on each sample was evaluated using Eq. (3.2).

$$\text{Cell proliferation ratio (\%)} = \frac{OD_{sample,1,3\ or\ 5d}}{OD_{TCPS,\ 1,3\ or\ 5d}} \times 100 \qquad (3.2)$$