CHAPTER III

RESULTS

3.1 Enumeration of *CD34*$^{+}$ cells from PBMCs cultures

The PBMCs were isolated using ficoll gradient centrifugation technique. Once suspension in RPMI 1640 or MEM medium, PBMCs are round shape and have the cellular diameter about 8 ± 2 μm (Figure 3.1a). The stem cells found in the PBMCs isolated were identified by positively staining with anti-*CD34*$^{+}$-FITC (Figure 3.1b). The enumeration of the anti-*CD34*$^{+}$-FITC positive cells was analyzed by flow cytometry (Figure 3.1c). By using our isolation technique, 0.1 % PBMCs was identified as PBSCs.

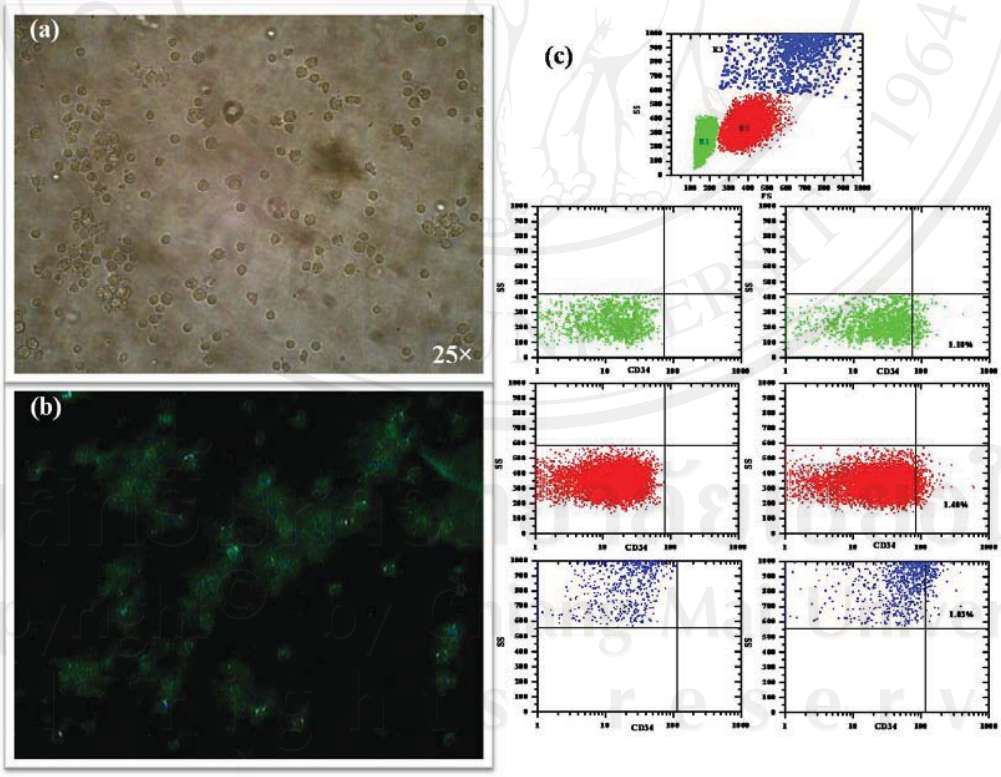


Figure 3.1 Characterization of peripheral blood stem cells by light micrograph (a), fluorescence micrograph (b) and cell counts using flow cytometometer (c).

3.2 Expansion of adult PBSCs in conventional culture

At high density such as 1×10^7 cells/mL, PBMCs could be maintained in RPMI 1640 and M199 medium at least 6 days without observation of any changes in cellular morphology. While the differentiation of cells and colonies of PBSCs were observed when the cultures were performed at lower density of PBMCs (varied from 10^5 to 10^6 cell/mL).

Figure 3.2 shows a progressive change of PBMCs. After seeding 10^6 cell/mL PBMCs into a culture at 24 hr, the cells were individually very rare aggregated (2-4 cells) distributed throughout the culture plates. The majority (80%) of PBMCs were found in oval shape (Figure 3.2a). Small colonies of cells about 3±2 colonies per well were observed. These cells were positively stained with anti-$CD34^+$-FITC corresponding to the so-called PBSCs. The PBSCs colonies were grown up as a function of culture time. Figure 3.2b shows an increase in colonies size and number after seeding the PBMCs in the culture for 6 days. As can be seen in Figure 3.2c some cells still suspended in the medium and some were found to attach onto the bottom surface of wells and the characteristic of PBSCs colonies defined as round cells at core and spindloid cells at the periphery were easily observed at day 8 after culture. When the cells were let in continuous culture for 24 days, the variety of cellular morphology were observed including spindle cells, rod and cone cells, foam cell, endothelial cells neuron-fiber etc. (Figure 3.2d). It should be noted that the differentiated cells were observed only for the attached cells.

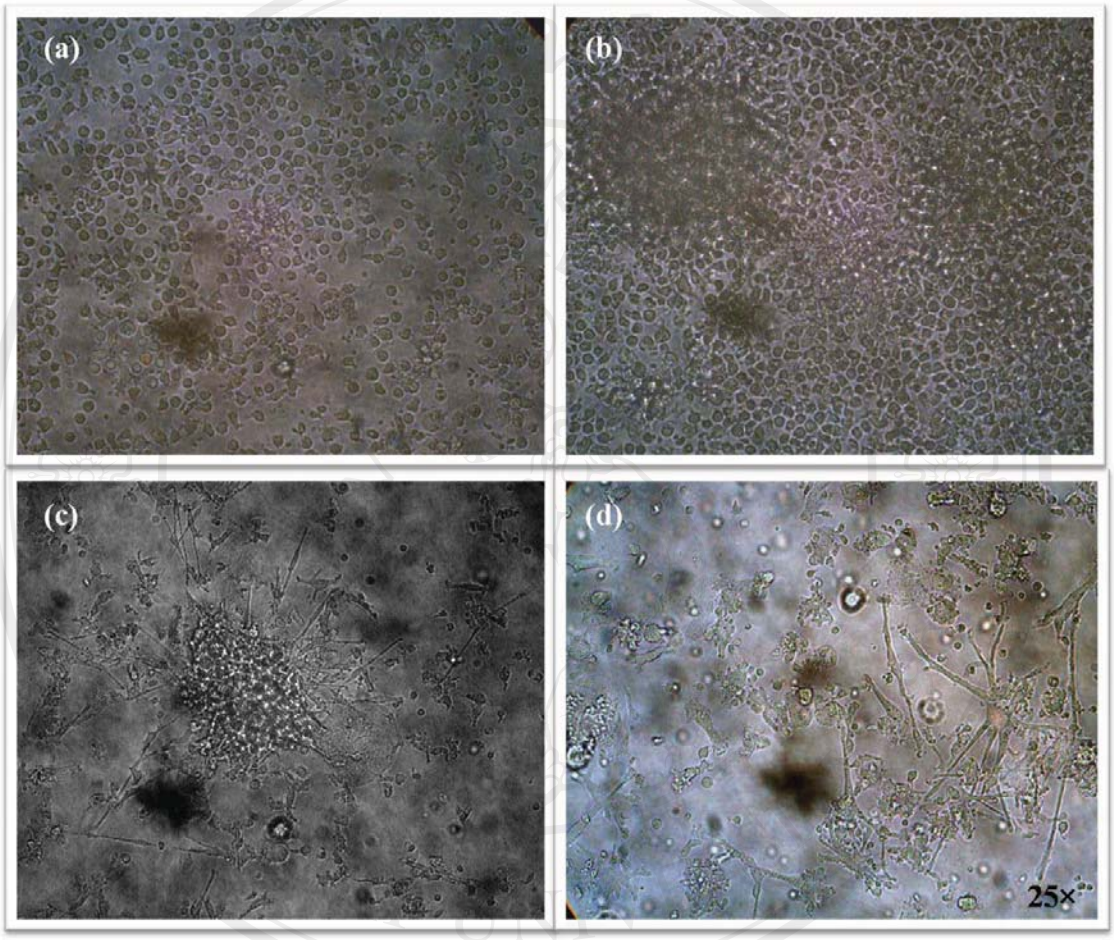


Figure 3.2 Micrograph of peripheral blood stem cells at day 1 (a), day 6 (b), day 15 (c) and day 20 (c) after seeded.

As previously mentioned the PBMCs were cultured in RPMI 1640 or MEM supplemented with 1 % penicillin-streptomycin and 10% fetal calf serum without any mitogens. The results signify that our conditions of experiments might be suitable for growing and promoting differentiation of the PBSCs. In fact, the PBMCs obtained in this study contain some amounts of platelets. We do a hypothesis that both platelets and PBMCs should act as extracellular matrix of the PBSCs.

3.3 Characteristic stem cell growth and division

The observation of cell morphology under an inverted light microscope provides evidence of colony forming units of stem cells in the culture system but it was difficult to enumeration of the stem cells. For the purposes of characterizing the growth pattern of these stem cells, two series with the same conditions of experiments were performed. One series the cells were observed under inverted microscope before trypsinization and the number of PBMCs was counted by heamocytometer.

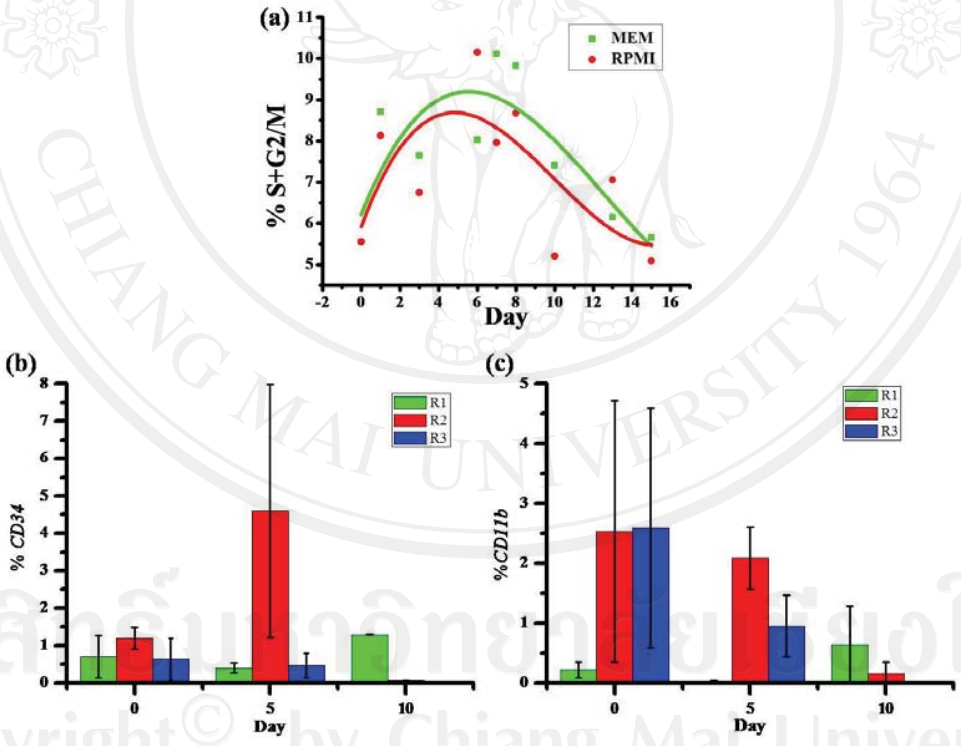


Figure 3.3 Growth and differentiation of PBSCs: growth pattern (a), variation of percentage of $CD34^{+}$(b) and $CD11^{+}$(c) cells as a function of culture time.

The cells were grown in RPMI 1640 and MEM medium the numbers of cells were increased by the days of culture and the maximum numbers were found at day 6 of

culture following a decrease (Figure 3.3a). It should be noted that at day 6 of culture, an increased in attached cell numbers and variety of morphology seems to be equilibrate with the suspended cell fraction. This might cause an error of number cell counts.

The other series of experiments, the cells were stained with anti-*CD34*$^{+}$-FITC and anti-*CD11b*-FITC as a function of culture time prior to analysis by flow cytometry. As can be seen in Figure 3.1c the cells obtained after letting in culture system divided in 3 subgroups assigned as R1, R2 and R3 region. Figure 3.3b showed that the *CD34*$^{+}$ cells which corresponding to PBSCs were initially found almost the same percentage in the three regions. At day 5 of culture the PBSCs in R2 was increased by 4 times of the initial condition then dramatically decreased at day 10. Contrary the PBSCs in R1 decreased by 50% at day 5 and increased at day 5. It should be noted that the PBSCs in R3 decreased and disappeared at day 10. Figure 3.3c demonstrated that the *CD11b*$^{+}$ cells which corresponding to monocytes and dentritic cells represent the mature cells were initially found in R2 and R3 region, these cells were decreased as a function of culture time and did not find in the culture at day 10. These results strongly suggested that there is an existing of kinetic status and homeostasis of PBSCs in the culture system and the life-span of mature cells should be shorter than 10 days.

In order to get further insight the cellular kinetic status, the DNA contents of the cells were analysized as a function of culture time by using PI in a flow cytometer. The histogram of DNA contents of cells was reported in Figure 3.4 As can be seen, at initial time of culture almost of cells were found in R1 where almost the cells were found in G0/G1 phase (94%) and the cells in R2 and R3 were found in S, G2 and M phase (5.4%).

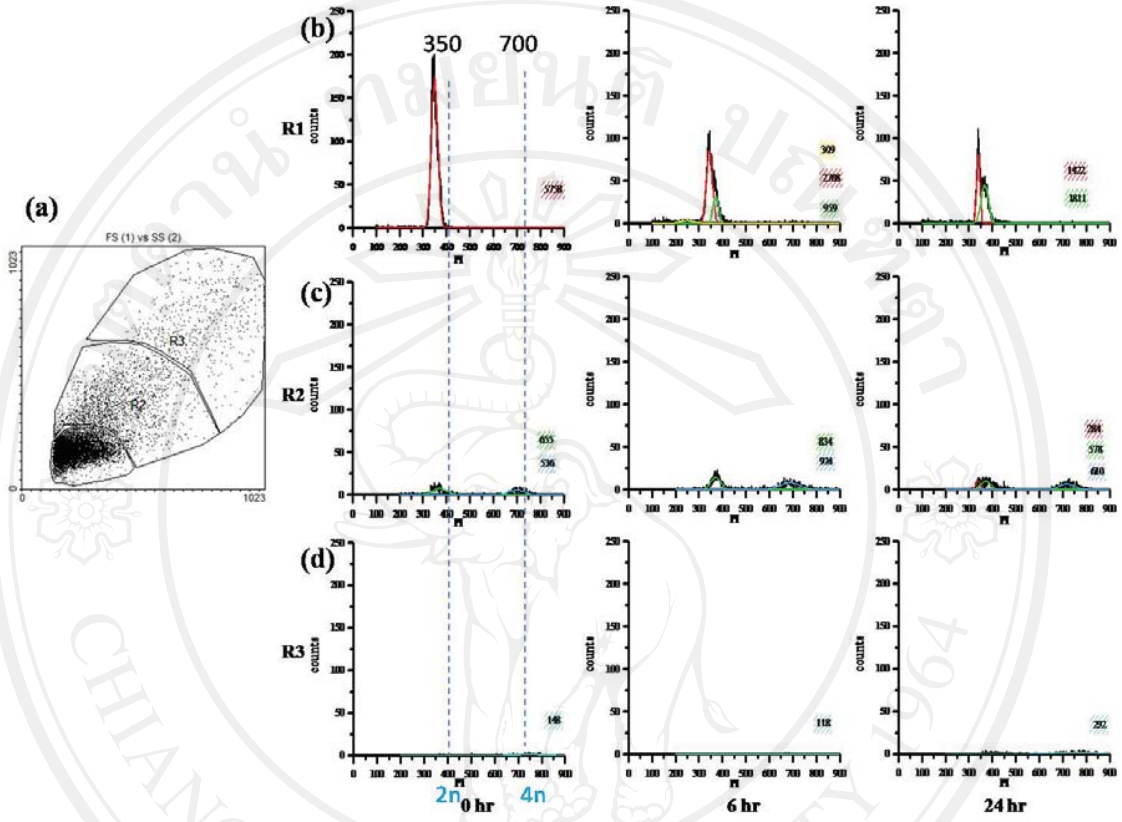


Figure 3.4 Flow cytometric analyses of DNA contents: light scattering characteristic of cells (a) and histogram of Cell bound PI fluorescence intensity of R1 (b), R2 (c) and R3 (d) region as a function of culture time.

The content of DNA of cells in R1 was found as Gaussian distribution and perfectly fitted using Gaussian equation (mode of PI fluorescence intensity = 350 au and half peak of maximum = 40 au). After 6 hours of culture, two new Gaussian peaks were appeared. One peak, the mode of PI fluorescence intensity shift to right hand of x-axis. The amplitude and shoulder of new peak was increased as a function of time. The results suggested that the cells leave from G1 and are entering to S phase of cell cycle. The other

peak, the mode of PI fluorescence intensity shift to left hand of x-axis indicating the cells in sub G0 phase corresponding to apoptotic cells by which obviously determined at 3 days after culture. This signified that PBSCs always maintain themselves or in other words, the number of PBSCs at steady state should be stable in our system.

3.4 Reconstitute cell communities and tissues of PBSCs on PVDF 3D-nanofibrous scaffold

In this series of experiments, the in house made of disc-like scaffolds with 250 mm diameter and 0.5 mm thickness, were used. It was verified that 3×10^6 cells in 50 μL of RPMI 1640 was the most appropriated conditions that can allow the cells to spread throughout on the scaffold surface. After letting the cells contacted to the scaffolds for 24 hours at 37°C in a CO_2-incubator, the scaffolds were transferred to new 6-well plates and 3 mL fresh RPMI 1640 medium were added and the cultures were further incubation for at least 90 days and the culture medium was changed at 4 days interval. It should be noted that the suspended colonies and attached cells outside the scaffolds always present. The attached cells on the well bottom under grew differentiation in similar way that observed in conventional culture technique.

Figure 3.5 demonstrated that the cells attached to the scaffold in both non-woven and alignment zone but different in morphology (Figure 3.5a and 3.5b). Almost the attached cells on the alignment zone were found to be differentiated in a variety of cellular morphologies when the cultures were maintained for 30 days and 60 days (Figure 3.5c and 3.5d). In order to get further insight the interaction between the fibers and cells, the scaffolds were analyzed using SEM and EDX. The micrographs also showed that the

fibers were coated with small vesicles probably containing proteins (Figure 3.6a). The most

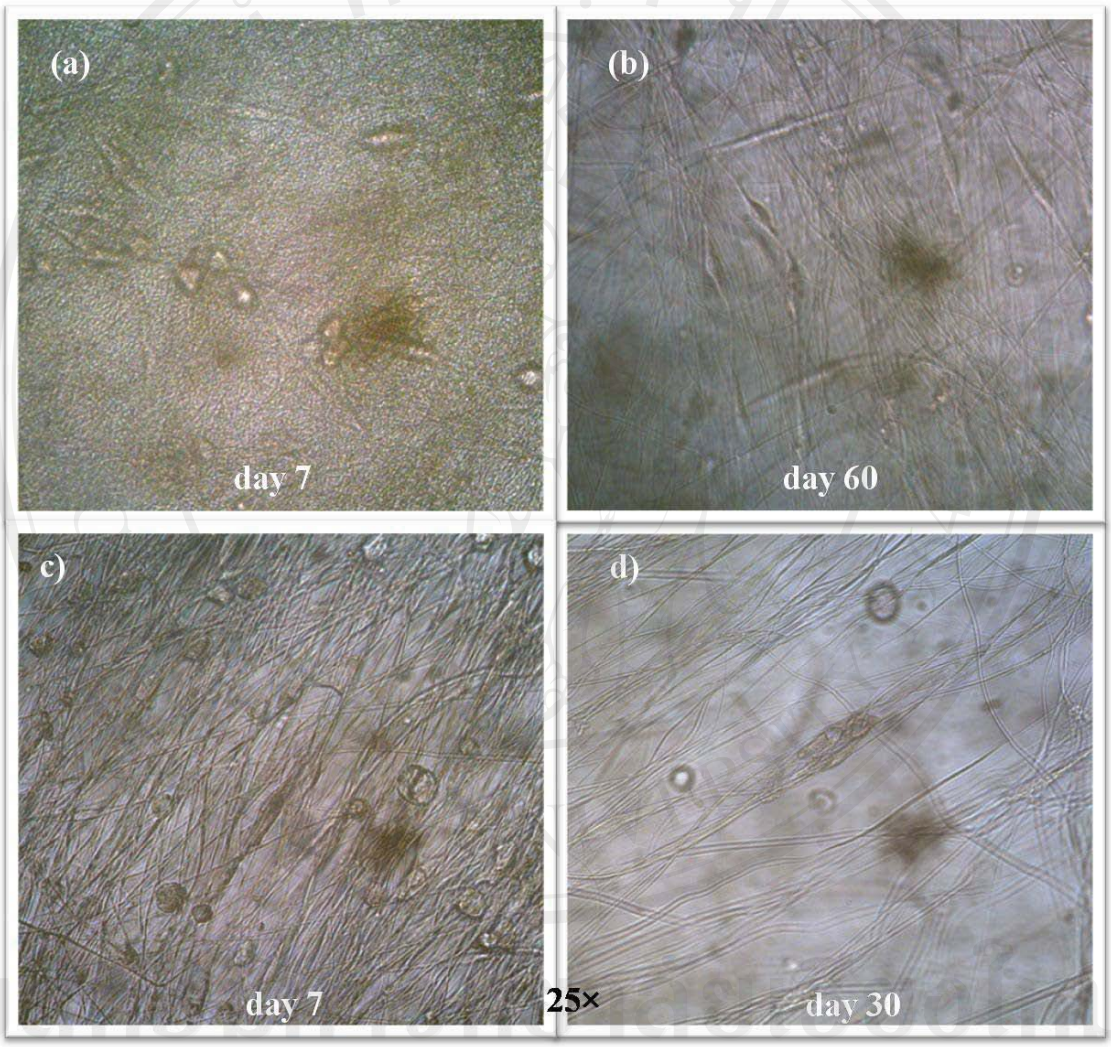


Figure 3.5 Micrographs of cells attached onto non-woven zone at day 7 (a) and day 60 (b) and aligned zone at day 7 (c) and day 30 (d) after seeded onto the top surface of 3D-PVDF nanofibrous scaffolds.

important data obtained from the micrographs were the irregular forms of cell organizations; vascular system and muscle bundle-like used the fibers as support for

hanging themselves in the space (Figure 3.6 a, b, c and d). These suggested that the cells need the 3 dimensions for organizing their own communities.

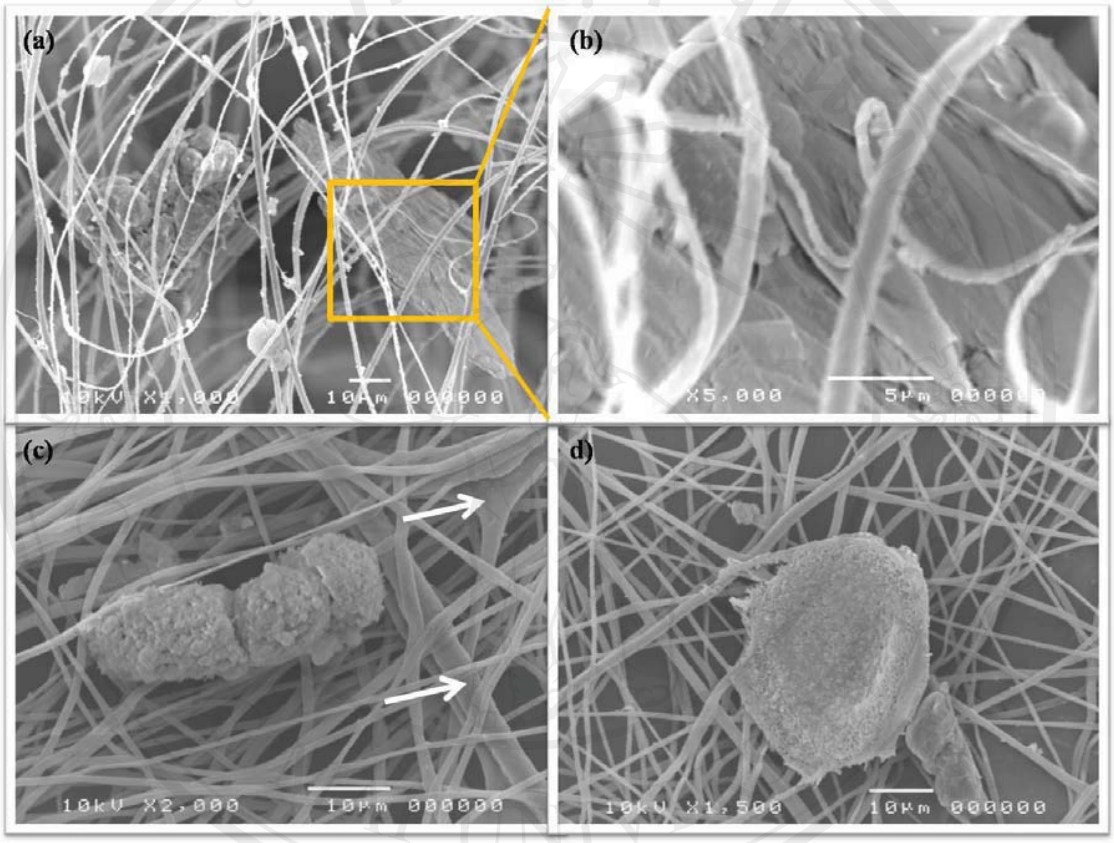
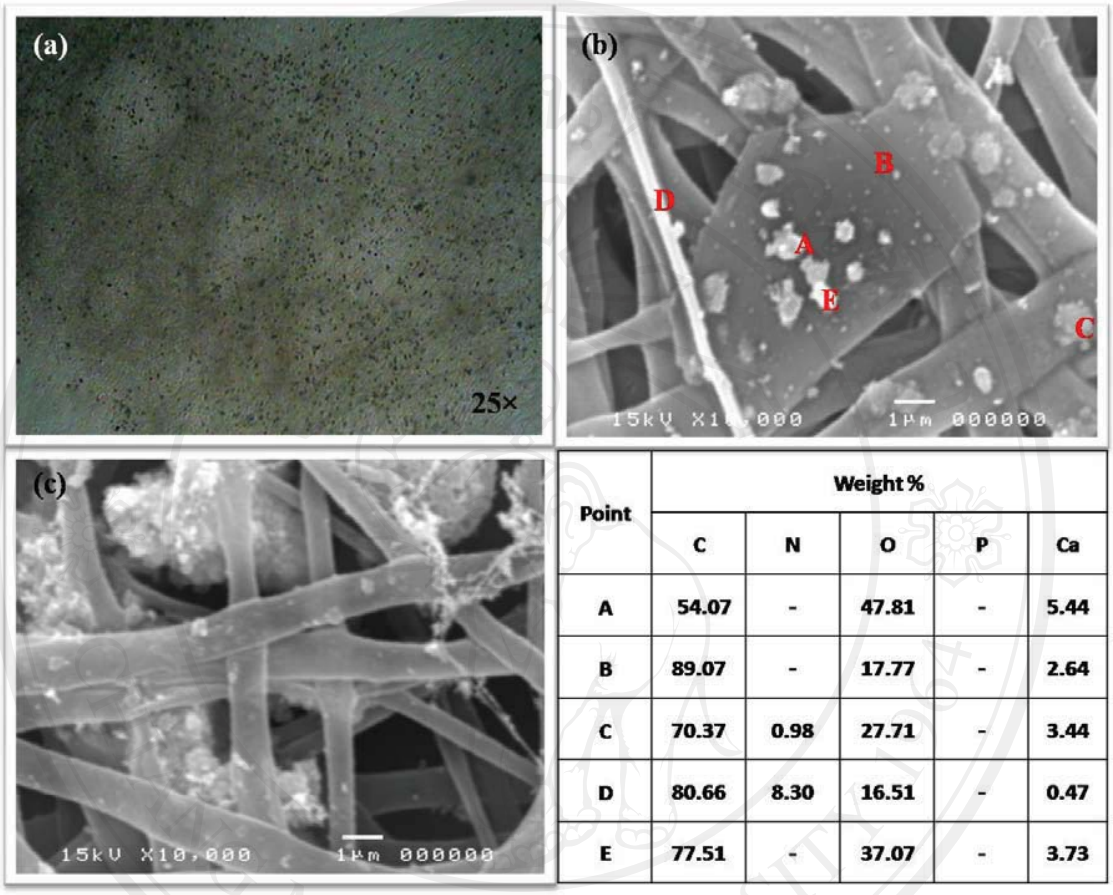


Figure 3.6 SEM Micrographs of cells attached onto the 3D-PVDF nanofibrous scaffolds at day 60

Indeed, the non-woven zone of the scaffolds was highly porous matrices and the diameter of pore site was average in micrometer range. These should be influenced on the attachment and growth pattern of the cells. As demonstrated in Figure 3.7 the photo micrographs of revealed that a small black spots spread throughout the scaffold (Figure 3.7a). These black spots were further characterized by SEM and EDX as indicated in Figure 3.7 b, c and d. Using high magnification of 10,000×, the spots consist of cells,



Point	Weight %				
	C	N	O	P	Ca
A	54.07	-	47.81	-	5.44
B	89.07	-	17.77	-	2.64
C	70.37	0.98	27.71	-	3.44
D	80.66	8.30	16.51	-	0.47
E	77.51	-	37.07	-	3.73

Figure 3.7 Characterization of bone tissue formation found in the non-woven zone of 3D-PVDF nanofibrous scaffolds at day 60 after cultured: photo micrographs of cells (a), high magnification SEM micrographs (b and c) of the same sample of Figure 7a and elements analysis of the indicated points of Figure 7b (d).

extracellular matrix such as and protein fibers connect to micro vesicles containing proteins and complexes of calcium. The cells found in the non-woven zone were irregular in shape and sites. The giant cells contain small fine dark granules concentrated in the perinuclear zone were found distributed throughout the scaffold (Figure 3.8a and b). Most

of the cells were positive stained with anti-*CD34*$^{+}$-FITC (Figure 3.8c and d). In the non-woven zone of the scaffolds, there were colonies of cells which have dark-blue color

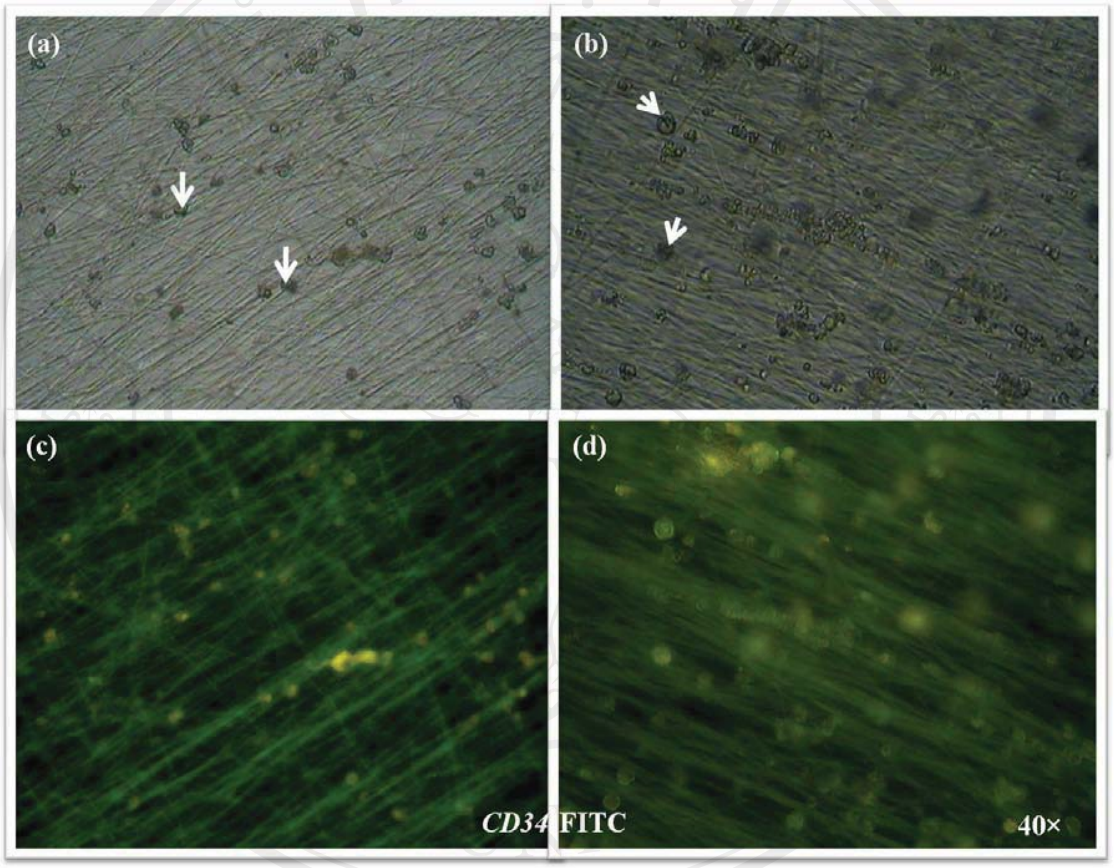


Figure 3.8 Micrographs of cells stained with anti-*CD34*$^{+}$-FITC: bright field of photo micrographs(a and b) and fluorescence micrographs of the same microscopic field at day 60 (c and d).

inside known as giant cells and mesenchymal stromal cells (Figure 3.9a). Figure 3.9b and c showed small cells of varying shape, irregular, polygonal, oval, and round with small nucleus adhere to the surface of the complex of giant and mesenchymal stromal cells. These cells were recognized as hematopoietic stem cells. This signified that most of

them should preserve the properties of stem cells. The results were confirmed by SEM and EDX experiments.

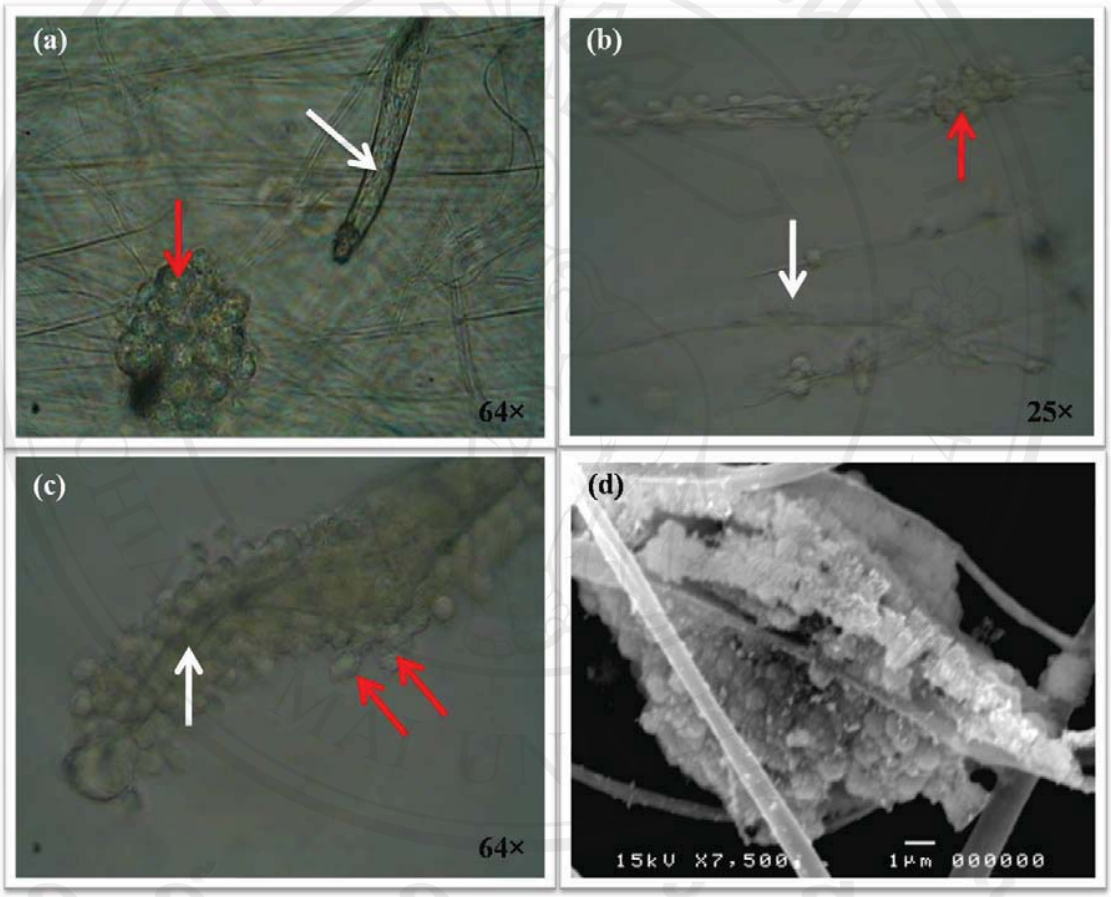


Figure 3.9 Micrographs of cells which cultured on the 3d-PVDF nanofibrous scaffold at day 60: (a, b and c) bright field photo micrographs of giant (red arrow) and stromal (white arrow) cells and (d) SEM micrograph of hematopoietic tissue.