

## **Fabrication of coated - collagen electro-spun PHBV nanofiber film by chemical method and its cellular study**

**J. Ai<sup>1</sup>, F. Ghorbani<sup>2</sup>, S. Heidari K<sup>3</sup>, E. Biazar<sup>4,\*</sup>**

<sup>1</sup>Department of tissue engineering, Faculty of advance technologies, Tehran university of medical sciences, Tehran, Iran

<sup>2</sup>Department of biomedical engineering, Science and research branch-Islamic azad university, Tehran, Iran

<sup>3</sup>Stem cell preparation unit, Eye research center, Farabi eye hospital, Tehran university of medical sciences, Tehran, and Clinical proteomics research center, Faculty of paramedical sciences, Shahid beheshti university of medical sciences, Tehran, Iran

<sup>4</sup>Department of chemistry, Islamic azad university-tonekabon branch, Iran

Received: 25 November 2010; Accepted: 16 January 2011

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### **Abstract**

Tissue engineering is defined as the designing and engineering of structures to rebuild and repair a body damaged tissue. Scaffolding Poly Hydroxy Butyrate Valerate (PHBV) has shown good biocompatibility and biodegradable properties. Nanofibers have improved the performance of biomaterials, and could be considered effective. One of the important methods for designing nanofiber scaffold is the electrospinning method. In this study, PHBV nanofibers were well designed; then, modified with the immobilized collagen via the chemical method. The samples were evaluated by ATR-FTIR, SEM, contact angle and finally, cell culture. ATR-FTIR structural analysis showed the presence of collagen on the nanofiber surfaces. The SEM images showed the size average of nanofibers as to be about 280 nm; that increased with a collagen coating up to 400 nm. Contact angle analysis showed 67 degree for uncoated nanofibers and 52 degree for coated nanofibers. Cellular investigations (USS cells) showed better adhesion and cell growth and proliferation of coated samples than uncoated samples.

**Keywords:** PHBV, Nanofiber, Collagen coating, Glutaraldehyde, Cellular investigation

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### **1. Introduction**

Poly Hydroxy Butyrate Valerate (PHBV) is a biomaterial that is used in a variety of applications including surgical sutures, wound dressing, drug delivery and tissue engineering. This is due to its specific properties such as good biocompatibility, biodegradability, non toxicity as well as its piezoelectricity features.

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\* Corresponding author: Esmail Biazar  
Islamic Azad University – Tonekabon  
Branch, Mazandaran, Iran  
Tel +98 1924274415, Fax +98192 4274409  
Email [E.biazar@tonekaboniau.ac.ir](mailto:E.biazar@tonekaboniau.ac.ir)

However, this material is a hydrophobic polyester which should be modified with other materials until it improves its cell adhesion and hydrophilicity properties [1,5]. It has been generally accepted that extracellular matrix mimics may improve the attachment, proliferation, and the viability of the cultured cells [6]. Electro-spinning has been rapidly developed into a technique to prepare nanofibers with the diameter ranging from tens of nanometers to several microns [7,9].

The electro-spun fibrous mats also show extremely high surface area and large porosity. Besides, the fibrous structure of the electro-spun mats may mimic the extracellular matrix. It is well-known that the collagen is a natural extracellular matrix component of nearly every tissue such as bone, skin, tendon, ligament, and so forth. Fiber diameters of the electro-spun nanofibrous mats even approach that of collagen fiber bundles, between 50 and 500 nm [9]. Therefore; during the last few years, many works considering the tissue engineering of electro-spun nanofibers have been reported. Most recently, electro-spun nanofibers were prepared by Yang et al. and were applied in neural tissue engineering [10]. Although the presented nanofibers may mimic the morphologies of extracellular matrix to some extent, some modifications are still required to create a friendly environment for the cells' attachment, proliferation, and functions such as communications. Some natural materials such as collagen, fibronectin and some peptides have been reported as scaffold modifiers [11,12]. Controlling surface properties is very important for the high performance of adhesion. Biomaterials wettability is an important factor in the surface modification of materials. Surface modification of hydrophobic polymer surfaces can be achieved by wet (acid, alkali), dry (plasma) and radiation treatments (ultraviolet radiation and laser) [13,16]. Non thermal and low pressure plasma have been used in a series of surface treatment applications. The majority of plasma-assisted technologies are based on low pressure processes [17]. The treatment of polymeric materials with plasma is a frequently-used technique to accomplish surface modifications that affects chemical composition as well as surface topography. Moreover, microwave discharges are routinely employed in the processing of materials to deposit films as well as coatings [18,19]. In this study, the USS cells were used. The USSCs were first isolated from the umbilical cord blood in 2003 by Jager et al., and their differentiation capacity for transplantation was evaluated [20]. Koghler et al. (2004) also evaluated these cells for cytokine production. The USSCs are pluripotent, also, they are considered as the rare cell populations in umbilical cord blood. They have a high potential to proliferate and differentiate, thus, they are considered as valuable sources in cell therapy [21]. The USSCs are CD45<sup>-</sup>, the adherent and HLA class II- negative stem cells with a long telomerase. Moreover, these cells possess a unique cytokine profile and have a high percentage of productions associated with self-renewing factors. In spite of other cord blood-derived mesenchymal cells, differentiated only into osteoblasts, chondrocytes, adipocytes [22], and neurons [21], the USSCs have the differentiation potential into osteoblasts, chondrocytes, blood cells, neurons, hepatocytes, and heart tissues under *ex vivo* conditions [21]. This cell expresses different factors, including adherent cells, growth factors, and various cytokines such as SCF, VEGF, GM-CSF, M-CSF, TGF-1 $\beta$ , IL-6, G-CSF, LIF, Flt3 ligand, TPO, HGF, SDF-1 $\alpha$ , IL-15, IL-12, IL-8, and IL-1 $\beta$  [22].

In this study, the coated collagen PHBV nanofibers were obtained through the chemical method. The samples were evaluated by ATR-FTIR, SEM and the contact angle also the cell culture with two USS cells.

## 2. Materials and methods

### 2.1. Materials

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) containing 5 mol% of 3-hydroxyvalerate with 680,000 molecular weight was purchased from Sigma Chemical Co. 2,2,2-trifluoroethanol (TFE) to prepare PHBV solution was purchased from Sigma-Aldrich Chemical and used as received without further purification.

### 2.2. Nanofiber preparation

Electrospinning apparatus used in this study prepared from asia nanomeghyas company (iran). PHBV was dissolved at determined concentration in TFE. The PHBV solution (2%w) is contained in a glass syringe controlled by syringe pump. A positive high voltage source through a wire was applied at the tip of a syringe needle. In this situation a strong electric field is generated between PHBV solution and a collector. When the electric field reached a critical value with increasing voltage, mutual charge repulsion overcame the surface tension of polymer solution and electrically charged jet was ejected from the tip of a conical shape as the Taylor cone. Ultrafine fibers are formed by narrowing of the ejected jet fluid as it undergoes increasing surface charge density due to evaporation of the solvent. Electrospun PHBV nanofibrous mat was carefully detached from collector and dried in vacuo for 2 days at room temperature to remove solvent molecules completely. Used parameters for this nano fibers preparation, as can be seen in [Table 1](#).

**Table 1.** Used parameters for nano fibers preparation

Syringe Diameter (mm)	dram speed (rpm)	Injected speed (mL/min)	Syringe tip distance to deram (mm)	Voltage (kv)	temperature (°C)	time (h)
17	1000	2	75	20	30	7

### 2.3. Collagen Immobilization

Collagen (type I, from bovine Achilles tendon, purchased from Sigma) was immobilized onto the nanofiber surface based on the following protocol. A weighted amount of the nanofibrous mat was rinsed with acetic acid buffer solution (50 mM, pH 5.0). Then, the mat was submerged into the collagen solution (15 mg/mL in acetic acid buffer solution, 50 mM, pH 5.0) and was shaken gently for 2 h at 50°C. The samples were exposure to glutaraldehyde 12% in hot water bath (80°C). The obtained samples were placed inside a vacuum oven so that they would fully lose humidity.

### 2.4. Fourier Transmission Infrared Spectroscopy

The samples were examined by Fourier Transmission Infrared Spectroscopy (FTIR; Bruker-Equinox 55; Bruker Optics, Billerica, MA) before and after adjustment. The samples were scratched into powder and were produced as capsules using KBr, and then, were put to investigation.

### 2.5. Scanning Electron Microscopy

The surface characteristics of various modified and unmodified films were studied with the help of the Scanning Electron Microscopy (SEM; Cambridge Stereo-scan, model S-360;

Cambridge Instruments, Wetzlar, Germany) to analyze the changes in the surface morphology. The films were first coated with a gold layer (Joel fine coat, ion sputter for 2 hours) to provide surface conduction before their scanning.

## **2.6. Contact Angle Analysis**

The sample surfaces static contact angles were investigated by a contact-angle-measuring device (Krüss G10; Krüss, Matthews, NC) following the sessile drop method.

## **2.7. Cellular Analysis**

### **2.7.1. Culture and Isolation of the USSC from Fresh Umbilical Cord Blood**

After taking consent from mothers, their umbilical cords were obtained from the cord vein. Only 40% of the cord blood samples contained USSCs. The mean age of donors was 28 years. After collecting the samples, the RBC was lysed using ammonium chloride (NH<sub>4</sub>CL) and the isolation procedure continued by Ficoll. Then, the samples were rinsed twice with sterile PBS (pH 7.4). After centrifuging, the resultant cells were placed in DMEM low glucose, which had been enriched with 100 nm dexamethasone, 10% FBS, penicillin, and streptomycin anti-biotics. The first medium exchange process was done for 24 h, then, every 4 days. When 80% of the flask surface area was covered by the cells, the cells were passed by using 0.25% Trypsin and EDTA. The USSCs were regularly expanded on the culture medium; and the 37°C temperature and 5% of CO<sub>2</sub> were required for the growth. The USSCs were first trypsinized and counted. The tubes, containing 10<sup>5</sup>-10<sup>6</sup> cells, were incubated on a rocker rotator for 10-6 h, centrifuged at 1000 rpm for 6 min, and 3% human serum was added to cell deposition thereafter. The resultant mixture remained at room temperature for 30 min. The cells were again centrifuged as above and the PBS was added to the cell deposition.

The cell mixture was passed through a nylon mesh, then, 100 µl of cells was added to each tube with the following antibodies: anti-CD<sub>90</sub>, anti-CD<sub>105</sub>, anti-CD<sub>166</sub>, anti-CD<sub>45</sub>, anti-CD<sub>73</sub> and anti-CD<sub>34</sub>. Next, they were kept at 4°C out of light for 45 min. After washing, the cells were fixed in 100 µl of 1% paraformaldehyde. Finally, the flow cytometric analysis was carried out. After and before co-culturing the mESC with the USSC, the Karyotype analysis was performed on both cell types. The first and last passages were preferably chosen for the Karyotype analysis. The cells were first placed in an incubator with 0.1 µg/ml colcemid for 3 to 4 h. Next, they were trypsinized; and 0.075 of M KCL solution was added to them thereafter. The cells were incubated with 5% CO<sub>2</sub> at 37°C for 20 min. In the next stage, methanol and acetic acids in a 1 to 3 ratio were added for fixing the samples. Finally, the cells were scattered over the slide surface and the chromosomes were subjected to Karyotype analysis.

### **2.7.2. Cell Culture Study on the Polymer Surface**

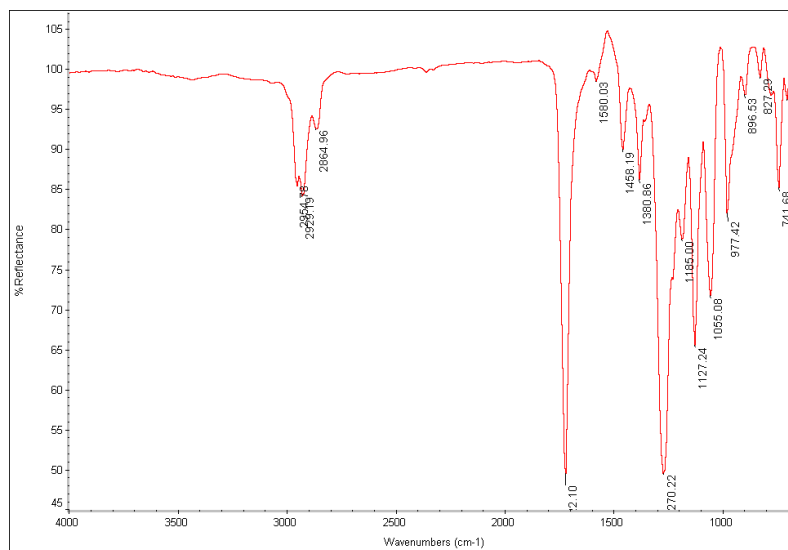
The powders to control (TCPS) were well cleaned and sterilized by the autoclave method. Individual samples were placed in Petri dishes using a sterilized pincer; The USS cells suspension was transferred to a flask (25°C) containing 5 mL of Dulbecco's modified Eagle's medium (DMEM; 2Mm l-glutamine, penicillin [100 lu/mL], streptavidin [100 L/mL]) and fetal bovine serum (FBS) 10%. The suspension was then placed in an incubator (5% CO<sub>2</sub>, 37°C). The fibroblast cells were proliferated in the flask and were washed using the FBS/ethylenediaminetetraacetic acid (EDTA). Then, the trypsin enzyme/EDTA was added to the flask (4°C); and the flask was incubated for 2 min. The culture media (FBS/DMEM) was added to the flask, and the cells were gently pipetted. The cell suspension was transferred to a falcon tube (15 mL) and centrifuged (1410 rpm) for 5 min.

The solution was removed and the precipitation was transferred to a new flask (75<sup>cc</sup>) for reculturing. Pieces of cell culture (1×1 cm) from the petri dish (control) and the main sample were placed individually in one of the Petri dish wells by using a sterilized pincer. 100,000 cells/well were seeded into a 24-well culture plate removed by pipette and were poured onto the control and the main samples. Then, all of the samples were placed in Memmert incubator at 37<sup>°C</sup> for 48 hours and were studied using a ceti microscope (wolf laboratories, UK). Cell proliferation was determined by the MTT assay for viable cell numbers. The MTT tetrazolium compound was reduced by living cells in a colored formazan product that was soluble in the tissue culture medium. The quantity of formazan product was directly proportional to the number of viable cells in the culture. The assays were performed by adding 1 mL of MTT solution (Sigma, St. Louis, MO) and 9 mL of fresh medium to each well after aspirating the spent medium and incubating at 37<sup>°C</sup> for four hours with protection from light. Colorimetric measurement of formazan dye was performed at a wavelength of 606 nm using a Rayto micro-plate reader.

### 3. Results and discussion

#### 3.1. FTIR Results

The results, from the ATR-FTIR spectrum of the uncoated nanofiber sample, the collagen film and the nanofiber sample modified with collagen, have been shown in figures 1-3. In **figure 1**, the strong band in 1720 cm<sup>-1</sup> has been shown to be related to the C=O group. The stretching band in 800-975 cm<sup>-1</sup> has been shown to be related to the -C-O-C- group and the stretching band in 2800-3000 cm<sup>-1</sup> has been shown to be related to the -CH<sub>3</sub> groups.



**Fig.1.** FTIR analysis of uncoated nanofiber film

**Figure 2** illustrates the ATR-FTIR spectra of collagen film. The spectra shows the strong band in 1720 cm<sup>-1</sup> as related to the C = O group, stretching bands in 2900-3000 cm<sup>-1</sup> as related to the -CH<sub>3</sub> groups. The stretching bands in 3318 cm<sup>-1</sup> and 3746 cm<sup>-1</sup> are related to the OH and NH groups.

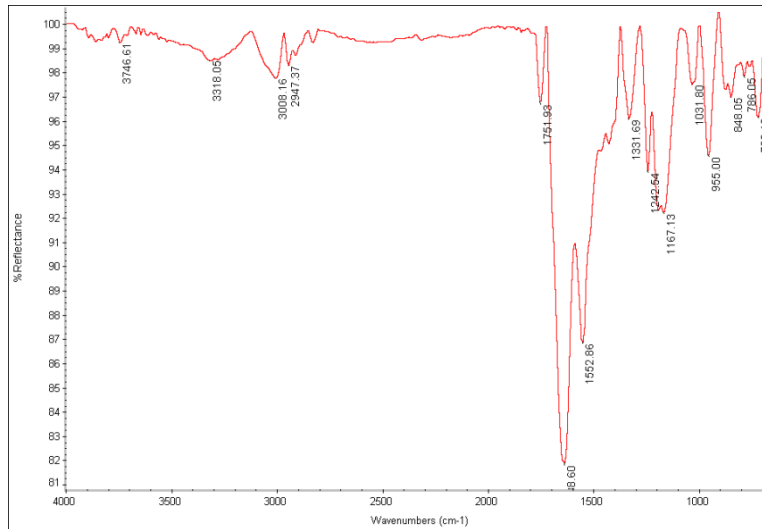


Fig .2. FTIR analysis of collagen film

Figure 3 also shows the strong band in  $1720\text{ cm}^{-1}$  as related to the C=O group , the stretching band in  $800\text{-}975\text{ cm}^{-1}$  as related to the -C-O-C- group and the stretching bands in  $2800\text{-}3000\text{ cm}^{-1}$  as related to the -CH<sub>3</sub> groups. The stretching band in  $3429\text{ cm}^{-1}$  and  $3783\text{ cm}^{-1}$  are related to the OH and NH groups due to the presence of collagen.

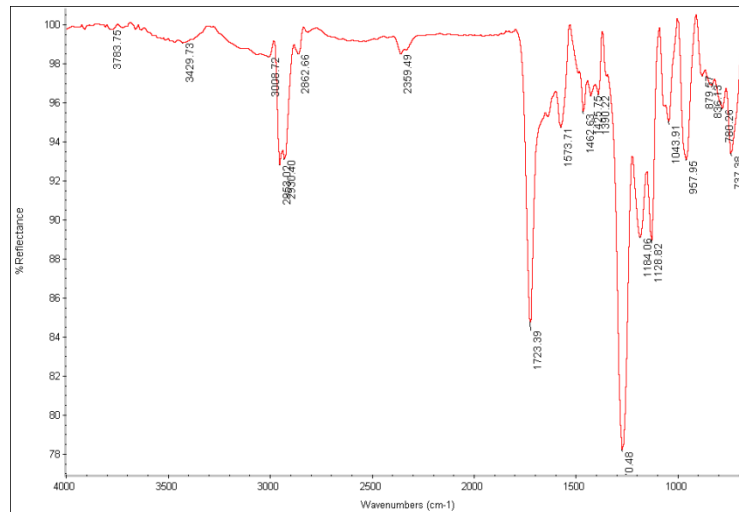


Fig .3. FTIR analysis of coated nanofiber film

### 3.2. SEM Investigations

The figures 4 and 5 show the Electron Microscopy Images of the uncoated and the coated nanofibers with collagen in different magnifications (4a; 1000x–4b; 10000x – 4c; 20000x). The smooth and homology nanofibers have been clearly shown in the figures. The size average was obtained for the nanofibers to be about 280 nm.



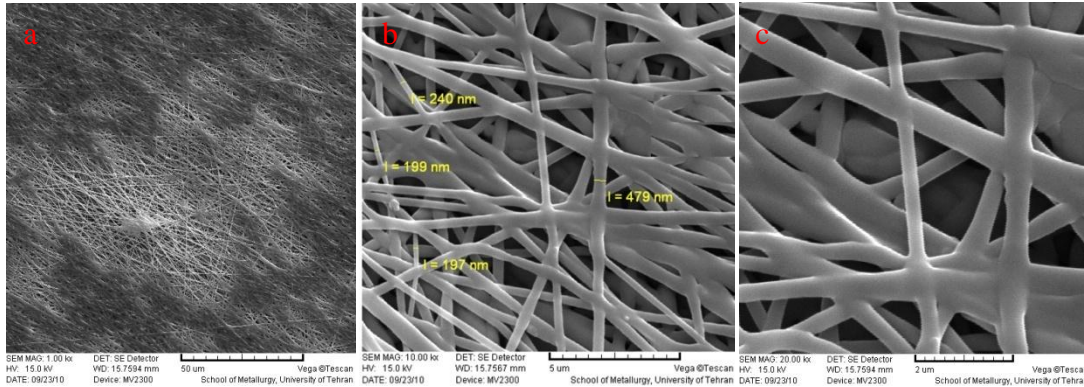


Fig.4. SEM images of PHBV nanofibers in different magnifications (4a; 1000x – 4b; 10000x – 4c; 20000x).

Figure 5 shows the collagen-coated-nanofiber mat prepared to the electro-spinning method of different magnifications (5a; 5000x – 5b; 10000x - 5c; 20000x). The smooth and homology Nanofibers have been clearly shown in the figures. The size average was obtained for the nanofibers to be about 400 nm, whose increasing is due to collagen coated on the PHBV surfaces.

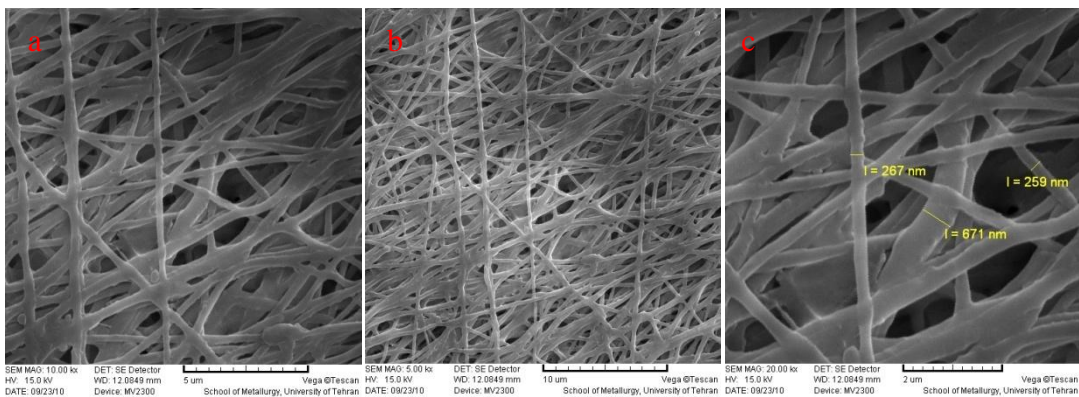


Fig.5. SEM images of coated collagen PHBV nanofibers in different magnifications (5a ; 5000x – 5b;10000x – 5c; 20000x).

### 3.3. Contact Angle Results

Table 2 shows the obtained contact angle for the uncoated and the coated collagen nanofibers. The contact angle of 67 degree and 52 were obtained for the uncoated and the coated nanofibers, respectively. The 15-degree of difference in the contact angle, obtained for the two samples, shows a better hydrophilicity of the collagen-coated nanofibers than the uncoated nanofibers.

Table 2. contact angle for uncoated and coated collagen nanofibers

T(°C)	PHBV nanofiber (uncoated) (degθ)	PHBV nanofiber (coated) (degθ)
25	67	52

### 3.4. Cellular results Cellular Results

The USSCs were isolated from the cord blood, and were then cultured. These cells were continuously cultured during 50 passages. The USSCs had a high proliferation capacity as they needed to repeat the passages. The proliferation and morphology of these cells were completely similar to each other before and after freezing. No indication of viral or mycoplasmal infection was observed during different stages of work on the USSC. The USSCs were morphologically adherent and spindle-shaped; they also had a size of 20-25  $\mu\text{m}$  (Figure 6).

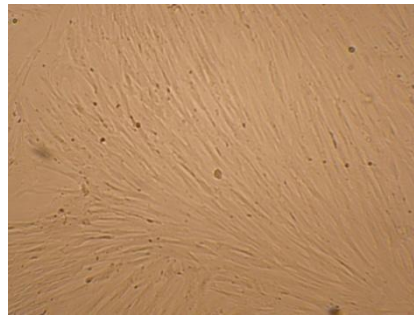


Fig.6. morphology of USSCs; spindle-shaped cells have a size of 20-25  $\mu\text{m}$

After 3 passages, the flow cytometric analysis was performed on the USSC in order to express the stem cell markers. The markers were as follows: CD<sub>34</sub>, CD<sub>45</sub>, CD<sub>73</sub>, CD<sub>105</sub>, CD<sub>90</sub> and CD<sub>166</sub> (Figure 7). For the USSCs, the expression of the surface markers like CD<sub>90</sub>, CD<sub>105</sub>, CD<sub>166</sub> and CD<sub>73</sub> was positive, but was negative for CD<sub>34</sub>, CD<sub>45</sub>.

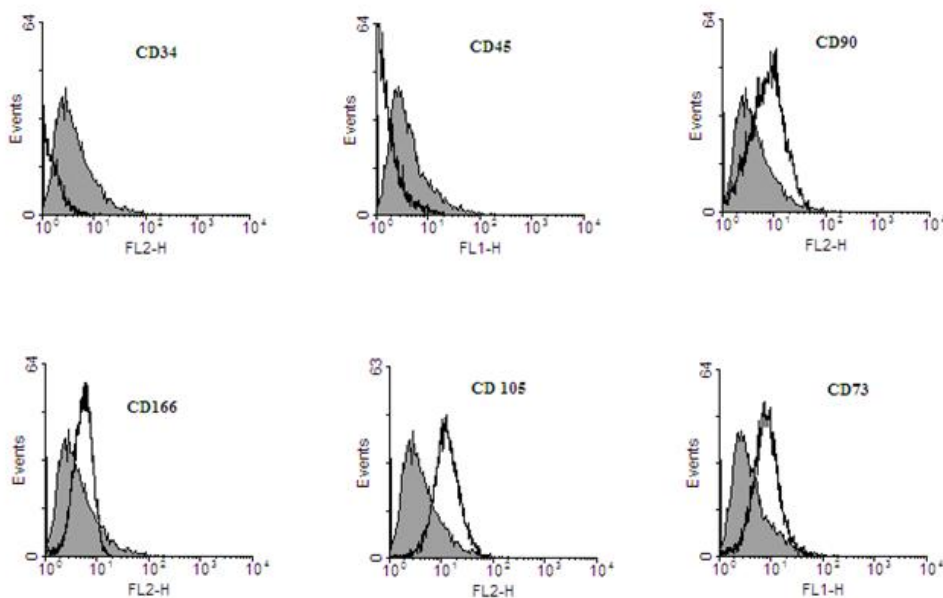


Fig.7. markers of flow cytometric analysis performed on USSC

Before beginning the experiments, the Karyotype analysis was performed on the USSCs of passage 2 which showed that they depicted a normal 44, XX Karyotype. After 48 passages, these



cells were subjected to the Karyotype analysis once again, and it was indicated that they had a normal chromosome Karyotype of 44, XX (Figure 8).

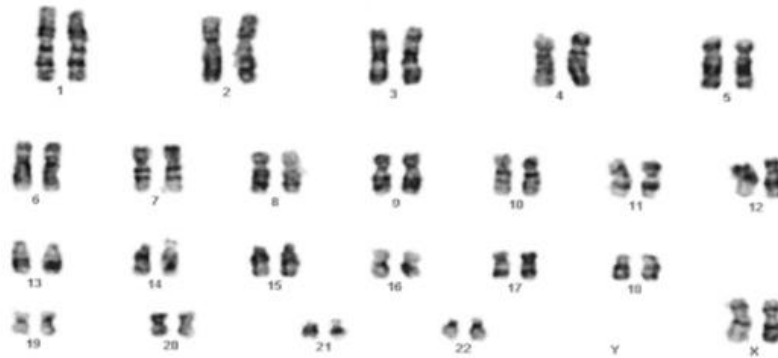


Fig.8. chromosome karyotype analysis performed on USSC

3.5. Cellular Study

Table 3 shows the MTT assay for TCPS (control), the uncoated nanofiber and the coated collagen nanofiber samples. The results showed a high viability for the samples of the uncoated nanofiber and the collagen-coated nanofiber (112, 130 % respectively), but the collagen-coated nanofiber showed a better viability than the uncoated nanofiber . Also, these samples caused more cells to proliferate. Figure 9 showed images of the cell culture on the nanofibers and the control sample. The image A is related to the control sample and the image b and c are related to the uncoated nanofiber and the collagen-coated nanofiber. Cellular images showed well growth in the vicinity of nanofibers especially the collagen-coated nanofiber.

Table 3. MTT analysis of the samples

Sample	$\lambda$ (nm)	Viability%
TCPS	606	100
PHBV nanofiber (uncoated)	677	112
PHBV nanofiber (coated)	792	130

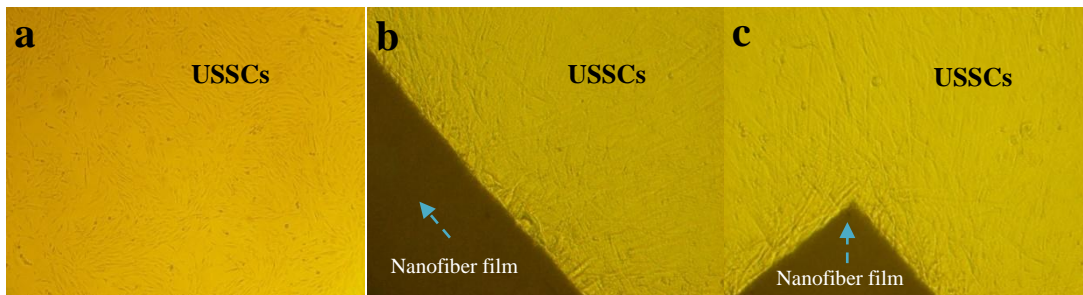


Fig.9. USS cell growth on the samples. Control a) TCPS b) uncoated PHBV nanofiber c) and coated PHBV nanofiber

#### 4. Conclusion

In this study, the PHBV nanofibers with a size average about 280 nm were designed. Nanofibers were successfully coated with collagen via the chemical methods shown in the analysis. A 15-degree difference in the contact angle, obtained for the two samples, showed a better hydrophilicity of the collagen-coated nanofibers than the uncoated ones. Cellular investigations showed better adhesion, growth and viability in the collagen-coated nanofibers than the uncoated nanofibers. These collagen-coated nanofibers could be used well for tissue engineering.

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