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Chapter 5

Fabrication of Silk Fibroin Nanofibres by Needleless Electrospinning

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65835

Abstract

Silk fibroin nanofibres were fabricated using a needleless electrospinning technique. The procedure focused on a new method for the preparation of a spinning solution from silk fibroin. The role of the concentration of silk fibroin solution, applied voltage and spinning distance were investigated as a function of the morphology of the obtained fibres and the spinning performance of the electrospinning process. The biocompatibility of the obtained fibre sheets was evaluated using an *in vitro* testing method with MG-63 osteoblasts. The solvent system consisted of formic acid and calcium chloride that can dissolve silk fibroin at room temperature, and a rate of 0.25 g of calcium chloride per 1 g of silk fibroin was required to obtain a completely dissolved silk fibroin solution. The diameters of the silk electrospun fibres obtained from the formic acid–calcium chloride solvent system ranged from 100 to 2400 nm, depending on the spinning parameters. Furthermore, increasing the concentration of the silk fibroin solution and the applied voltage improved spinning ability and spinning performance in needleless electrospinning. In addition, *in vitro* tests with living cells showed that the obtained electrospun fibre sheets were highly biocompatible with MG-63 osteoblasts.

Keywords: silk fibroin, needleless electrospinning, formic acid, calcium chloride

1. Introduction

Silk is a fibrous protein that is produced by a variety of insects, including the silkworm, and silk fibres from silkworms have been used in textiles for almost 5000 years. The primary

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reasons for this enduring use have been the unique lustre, tactile properties, high mechanical strength, elasticity, durability, softness and dyeability of silks. Silks also display interesting thermal and electromagnetic responses, particularly in the UV range, for insect entrapment, and form crystalline phases related to processing [1]. In addition to its outstanding mechanical properties, silk is a candidate material for biomedical applications, as it has high biological compatibility and oxygen and water vapour permeability, in addition to being biodegradable and having minimal inflammatory reactions [2–5]. Silks have historically been employed in medicine as sutures during the past 100 years, and are still currently used in this manner, as well as in a variety of consumer product applications. Commercially, silkworm cocoons are mass-produced in a process termed 'sericulture' [1]. Although the silkworm spins its cocoon from a continuous filament of silk, the remainder of the silk cocoon is unsuitable for reeling and is known as silk waste. Silk waste also encompasses waste silk from all stages of production, from reeling through weaving. The composition of silk waste is similar to that of good silk. It has been roughly characterised by scientists, and has shown a high level of remaining nutrients, such as protein and lipid, which can be transformed into high-value products, for example, cosmetics, medical materials for human health, and food additives [6].

The silk fibroin protein is a structuring molecule that can be processed into numerous forms using a variety of techniques. Several different material morphologies can be formed from aqueous or organic solvent formulations of the natural fibre form of silk for utilisation in biomaterials for biomedical applications. Natural silk fibres dissolve only in a limited number of solvents because of the presence of a large amount of intra- and intermolecular hydrogen bonds in fibroin and its high crystallinity. Dissolution methods used for solubilising degummed silk fibroin fibres generally rely on strong chaotropic agents, including concentrated acids (hydrochloric acid, phosphoric acid and sulphuric acid) and aqueous salt solutions in high ionic strength (such as lithium thiocyanate, lithium bromide, calcium chloride, zinc chloride and magnesium chloride) to neutralise the hydrogen bonds stabilising the silk crystal structure. Consequently, the conditions of the dissolution process can influence the chemical composition and the molecular structure of the silk protein, affecting its biomaterial properties [7, 8].

The most ubiquitous method of producing regenerated silk fibroin solution is through the use of heavy salts, such as LiBr or $CaCl_2$. The degummed fibres can be dissolved using LiBr (9.3 M) solution or a ternary solvent system of $CaCl_2/C_2H_5OH/H_2O$ (1/2/8 mole ratio) to disrupt hydrogen bonding between the fibroin protein chains. The solution should then be allowed to thoroughly dissolve for up to 4 hours at 60°C to ensure complete dissolution. The heavy salts can then be removed from the silk solution through dialysis against deionised water over a period of 72 hours. Typically, the molecular weight cut-off for the dialysis membrane is 3500 Da, which is sufficiently permeable to allow the salts and water to travel freely, while retaining the fibroin light and heavy protein chains, respectively. The main disadvantage of a salt-containing aqueous solvent is the long preparation time, as aqueous solutions of fibroin must be dialysed for several days in order to remove the salts and recover the polymer as films, sponges or powder from the aqueous solution by dry forming. In some organic solvents (e.g. hexafluoroacetone and hexafluoroisopropanol),

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silk fibroin can be dissolved only after preliminary activation by dissolution in aqueous salt systems followed by recovery [7–9].

The electrospinning of silk solution is a favoured processing methodology for producing nanometre- to micron-scale fibres that result in a high degree of available surface area for use in creating scaffolds for tissue engineering and regenerative medicine purposes [9]. Electrospinning technology can be divided into two branches: needle electrospinning and needleless electrospinning. The needle electrospinning setup normally comprises a high-voltage power supply, and a syringe needle connected to a power supply and a collector. During the electrospinning process, a high electric voltage is applied to the polymer solution. When the electric force overcomes the surface tension of the polymer solution, the latter is ejected off the tip of the Taylor cone to form a polymer jet [10, 11]. As a needle can produce only one polymer jet, needle electrospinning systems have very low productivity, typically less than 0.3 g/h per needle, making them unsuitable for practical uses [10]. However, needleless electrospinning systems have recently been developed, in which, instead of the generation of a polymer jet from the tip of the needle, polymer jets form from the surface of free liquid by self-organisation [11–19]. For example, Jirsak et al. [14] invented a needleless electrospinning system using a roller as the fibre generator. The roller electrospinning device contains a rotating cylinder electrode, which is partially immersed in a polymer solution reservoir. When the roller slowly rotates, the polymer solution is loaded onto the upper roller surface. Upon application of a high voltage to the electrospinning system, a number of solution jets are simultaneously generated from the surface of the rotating spinning electrode, thereby improving fibre productivity [10].

In order to utilise silk waste, as well as to achieve large-scale production of electrospun fibre sheets, the regeneration of silk fibres is necessary, via a simple, but efficient, spinning process. A needleless electrospinning technique was chosen for fabrication of silk fibroin electrospun fibre sheets in the present study, as a result of its capacity to fabricate nanofibre layers on a mass industrial scale. However, needleless electrospinning is a new technique, and the majority of previous research on electrospinning of silk fibroin has focused on the needle electrospinning system. There is little information relating to the electrospinning of silk fibroin with a needleless system; therefore, the parameters of the spinning process have not been identified. The aim of the present research was to fabricate silk fibroin nanofibres with a needleless electrospinning method, and the experiment intensively concentrated on the effect of various parameters on the electrospinning process. The role of the concentration of silk fibroin solution, applied voltage and working distance were investigated as a function of the morphology of the obtained fibres and the spinning performance of the electrospinning process. In addition, a new method for preparation of the spinning solution, namely, dissolving silk fibroin in a mixture of formic acid and calcium chloride, was used for solution preparation instead of a ternary solvent system of $CaCl_2$/C_2H_5OH/H_2O [20], which has been widely used to dissolve silk fibroin. Furthermore, characterisation of properties of the obtained electrospun fibre sheets and their interaction with living cells was also carried out.

2. Experiment

2.1. Materials

Waste cocoons of *Bombyx mori* Linn. Thai silkworm (Nang-Noi Srisakate 1) were supplied from Chan farm, Amphoe Mueang Chan, Si Sa Ket Province, Thailand, and waste silk cocoons, in the form of pierced cocoons, were obtained from the supplier. FBA-free ECE phosphate reference detergent (Union TSL Co., Ltd., Thailand) was used as a soaping agent in the degumming process. The chemicals used for the preparation of the spinning solutions were calcium chloride and 98% formic acid. All other chemicals used in this study were reagent grade.

2.2. Preparation of silk fibroin solutions

Silk fibroin (SF) was obtained using a degumming process. In brief, pierced cocoons were cleaned by first manually removing the contaminants and then washing twice with water. The cleaned raw cocoons were then dried in the sun. Raw silk cocoons were degummed twice with 1 M sodium carbonate (Merck Ltd.) and 0.5% of a soaping agent at 100°C for 30 minutes. Following degumming, the silk fibroin was washed in boiling water for 30 minutes to remove any remaining sericin from the surface of the fibre, and then washed again with warm water and dried at room temperature. Silk fibroin solutions were prepared by dissolving the degummed silk fibres in a mixture of formic acid and calcium chloride. The silk fibroin concentration varied from 6 to 14 wt%. All solutions were magnetically stirred at room temperature overnight.

2.3. Investigation of the effect of calcium chloride on the dissolution behaviour of silk fibroin in formic acid

In order to ascertain the appropriate amount of calcium chloride for dissolution of silk fibroin in formic acid, degummed silk fibres were directly dissolved in formic acid (98%) with various amounts of calcium chloride to prepare 12 wt% of silk fibroin solution. The weight ratio of silk fibroin to calcium chloride was 1:0.20, 1:0.25, 1:0.30, 1:0.35 and 1:0.40 (w/w), respectively. All solutions were magnetically stirred at room temperature and then electrospun into fibre sheets.

2.4. The needleless electrospinning process

A schematic representation of the equipment used in the spinning process is presented in **Figure 1**. The electrospinning device contained a rotating electrode, which was made of stainless steel wire of 125 mm in length and 0.02 mm in diameter (**Figure 2**), and a solution reservoir. The solution reservoir, which had a high voltage connected to the bottom of the solution bath, was filled with the silk fibroin solution. The process parameters are shown in **Table 1**. The electrospun fibres sheet was collected on the backing material moving along the collector electrode at a velocity of 10 mm/min.

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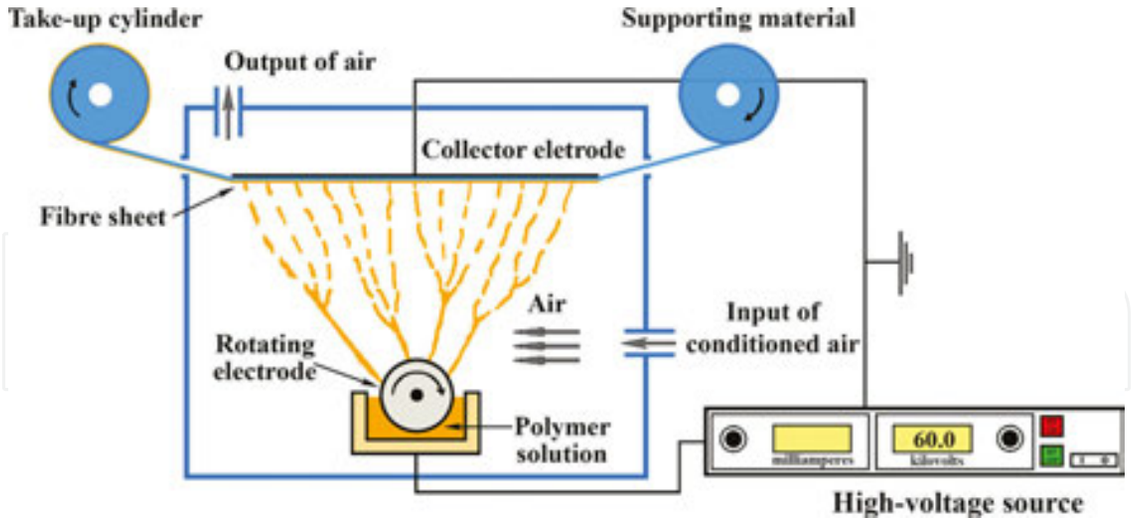

Figure 1. Schematics of the needleless electrospinning setup.

Figure 2. A spinning electrode.

Process parameters	Parameters level
Silk fibroin concentration (wt%)	6, 8, 10,12, 14
Applied voltage (kV)	30, 40, 50, 60
Distance between electrodes (mm)	100, 125, 150
Air humidity (%)	35–40
Temperature (°C)	20–25

Table 1. The spinning parameters of an electrospinning experiment.

2.5. Post-treatment of electrospun fibre sheets

Electrospun fibre sheets were treated with alcohol to achieve the solvent-induced crystallisation of the silk fibroin and to reduce the water solubility of the fibre sheets. The obtained fibre sheets were immersed in absolute ethanol for 30 minutes. After drying at room temperature, the treated fibre sheets were removed from the backing substrate and immersed in distilled water overnight, after which they were rinsed in distilled water to remove residual salts and then finally dried again.

2.6. Characterisation

2.6.1. Morphology analysis and fibre diameter

The morphological appearance of the electrospun fibres was observed with a scanning electron microscope (Vega 3, Tescan, Czech Republic) at an accelerated voltage of 20 kV. All the samples were sputter-coated (Q150R ES, Quorum Technologies Ltd., England) with gold at a thickness of 7 nm. The diameter of the nanofibres was measured by counting image pixels with NIS-Elements AR image software (LIM s.r.o., Czech Republic). The average fibre diameter and distribution were determined from 200 random fibres obtained under each spinning condition.

2.6.2. Spinning performance of the electrospinning process

Spinning performance is one of the most important parameters of the needleless electrospinning method. It was calculated from the mass per unit area and width of the electrospun fibre sheets and the velocity of the backing material, using the equation 1 [16]:

$$P = \frac{G \times W \times V_f}{L_r} \tag{1}$$

where P is a spinning performance (g/min/m), G is a mass per unit area of electrospun fibres sheet in grams per square metre (g/m^2), W is width of fibre layers in metres (m), V_f is take-up cylinder speed in metres per minute (m/min) and L_r is length of spinning electrodes in metres (m).

2.7. *In vitro* tests of silk electrospun fibre sheets

2.7.1. Preparation of scaffolds and cell seeding

The fibre sheets were cut into small disks with a diameter of 6 mm to fit each well of a 96-well plate. The specimens were sterilised by immersion in a 70% aqueous ethanol solution for 30 minutes, followed by double washing in phosphate-buffered saline (PBS, Lonza). MG-63 osetoblasts were cultivated in Eagle's Medium (Lonza) supplemented by 10% foetal bovine serum and 1% antibiotics. The cells were placed in a humidified incubator at an atmosphere of 5% CO_2 at 37°C. When they became confluent, the cells were suspended using trypsin-EDTA, centrifuged and re-suspended in fresh complete medium. The number of cells was determined by a Luna™ cell counter. Osteoblasts were seeded on the scaffolds and placed in a 96-well plate at a density of 5×10^3 per well plate. The medium was changed three times per week during the experiment.

2.7.2. Cell adhesion and proliferation analysis

The viability of the cells seeded on the scaffolds was analysed via MTT test after 1, 3, 7 and 14 days of culture. In brief, after cell culture, 50 μl MTT solution (Sigma Aldrich) was added to

150 μl of Dulbecco's Modified Eagle's Medium (DMEM) and the specimens were incubated at 37°C for 4 hours. When MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) was reduced to purple formazan by mitochondrial dehydrogenase in the cells, this indicated normal metabolism. The formed violet crystals of formazan were then solubilised with acidic isopropanol, and the optical density of the suspension was measured using an Absorbance Reader ELx808 (BioTek). For each testing day, four samples of each material were incubated with MTT solution and average absorbance was calculated as the difference between absorbance measured at 570 nm and by reference wavelength of 690 nm.

2.7.3. Fluorescence microscopy analysis

After the culture period, the samples were washed twice in PBS and fixed in frozen methanol for 30 minutes followed by double washing with PBS and staining with propidium iodide (PI dilution 2 g/l PBS, Sigma Aldrich) for 10 minutes in the dark. The stained cells were observed by inverted fluorescence microscope (Nikon).

2.7.4. Cell morphology

The cell morphology was analysed using a scanning electron microscope (SEM). After days of culture the samples were washed twice in PBS and fixed in 2.5% glutaraldehyde in PBS for 30 minutes at 4°C. The samples were then dehydrated by gradients of ethanol (60, 70, 80, 90, 96 and 100%, respectively). After water removal, the scaffolds were transferred to the SEM holder, sputter-coated with gold and observed under the SEM.

3. Results and discussion

3.1. The effect of calcium chloride on the dissolution behaviour of silk fibroin

3.1.1. The effect of calcium chloride on solubility of silk fibroin in formic acid

Figure 3 shows the difference in dissolution of silk fibroin in formic acid with varying amounts of calcium chloride. It was observed that silk fibroin was insoluble in formic acid, but was soluble in a mixture of formic acid and calcium chloride. This shows that calcium chloride can improve the solubility of silk fibroin in formic acid. It is suggested that calcium chloride has a chaotropic property that disrupts stabilising intra-molecular forces, such as hydrogen bonds, van der Waals forces and hydrophobic interactions, in protein structures by shielding charges. Hydrogen bonds are stronger in non-polar media. Calcium chloride that increases the chemical polarity of the solvent can also destabilise hydrogen bonding in the silk fibroin structure and ion-dipole interactions between the salts and hydrogen bonding species, which are more favourable than normal hydrogen bonds. This increases the solubility of hydrophobic proteins in the solvent [7, 21]. The results showed that silk fibroin completely dissolved in formic acid when the weight ratio of 1:0.25 (w/w) of silk fibroin to calcium chloride was used.

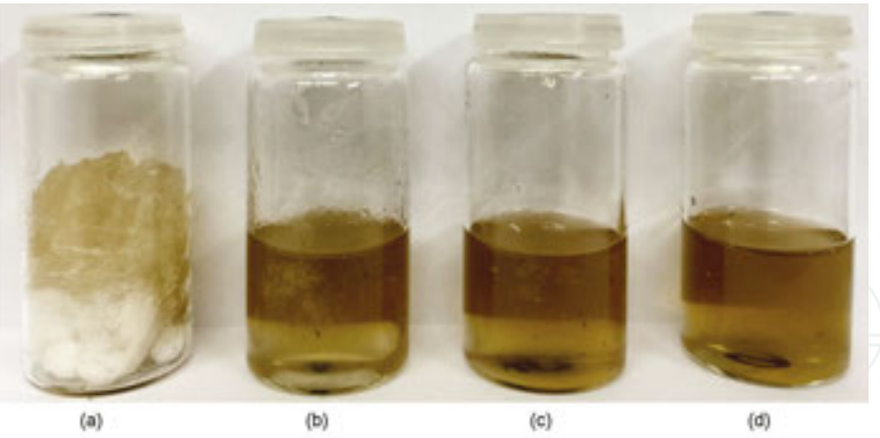

Figure 3. The dissolution of silk fibroin in formic acid with varying amounts of calcium chloride: (a) without $CaCl_2$; (b) 1:0.15; (c) 1:0.20; (d) 1:0.25 [SF:$CaCl_2$ (w/w)].

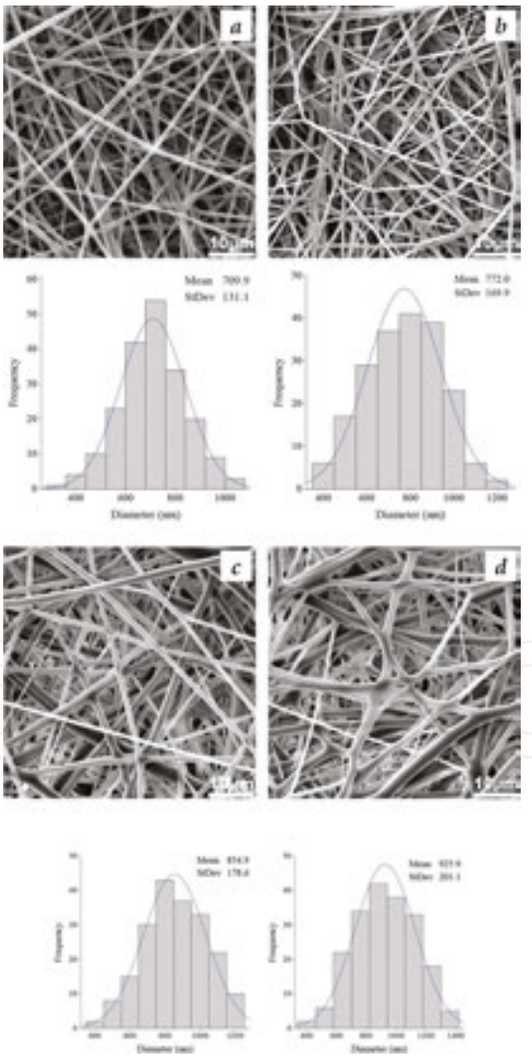

Figure 4. SEM micrographs and diameter distribution of electrospun fibres prepared from silk fibroin 12 wt% with varying amounts of $CaCl_2$: (a) 1:0.25; (b) 1:0.30; (c) 1:0.35; (d) 1:0.40; (SEM magnification 5×).

3.1.2. The effect of calcium chloride on the morphology of silk fibroin electrospun fibres

The SEM micrographs and diameter distribution of the silk fibroin electrospun fibres prepared from silk solutions with varying amounts of calcium chloride are shown in **Figure 4**. The results showed that the average fibre diameter increased with an increase in the amount of calcium chloride. Moreover, increasing the weight ratios of silk fibroin to calcium chloride affected the morphology of the obtained fibres; the fibres changed slightly from circular cross section to flat. It is assumed that the addition of a large quantity of calcium chloride can result in a change in the evaporation of solvent. Calcium chloride has the capacity to attract moisture from the air and surroundings, so the capacity of the silk fibroin solution to absorb water was increased when the amount of salt was increased. As a result, the solution absorbed more ambient water during electrospinning. The absorption of water does not allow completion of the drying process during the time of flight of the solution jet, and this phenomenon could lead to a slowing in evaporation of the solvent, which may result in an increase in fibre diameter and produce congealed mats instead of unwoven fibres [22, 23]. Therefore, the weight ratios of 1:0.25 and 1:0.30 (w/w) of silk fibre to calcium chloride appear to be suitable for the preparation of silk fibroin solution, which is used for an electrospinning process. The weight ratio of 1:0.25 (w/w) was eventually preferred and used throughout the remaining experiments.

3.2. The effect of parameters on needleless electrospinning of silk fibroin

3.2.1. The effect of silk fibroin concentration

The effect of concentration of the solution on fibre morphology with the applied voltage of 60 kV when the silk fibroin concentration increased from 6 to 14 wt% was considered. The effect of silk concentration on the morphological appearance and diameter of the electrospun fibres was investigated by SEM, as shown in **Figure 5**. It was found that an increase in the concentration of silk fibroin solution produced a significant effect on the average fibre diameter and the uniform diameter distribution of the obtained electrospun fibre. The results showed that the fibre diameter and non-uniform fibre diameter distribution of the obtained electrospun fibres increased with an increase in the silk fibroin concentration, showing the important role of the concentration of the silk fibroin solution in fibre formation during the needleless electrospinning process. When the concentration was increased from 6 to 14 wt%, the average fibre diameter increased from 191 to 1500 nm, respectively. The concentration of the polymer solution reflects the number of entanglements of polymer chains in the solution, which, in turn, affected the viscosity of the solution. An increase in the concentration of the silk solution resulted in greater polymer chain entanglement. Thus, the viscosity of the solution also increased. At higher concentrations, the diameter of the fibre was greater. In addition, the interaction between the solution and the charges on the jet determined the distribution of the fibre diameters that were obtained. This was probably due to the number of jets that formed during electrospinning. Multiple jets may have formed from the main electrospinning jet, which was sufficiently stable to yield fibres of a smaller diameter at certain concentrations, thereby generating fibres with various diameters [24, 25].

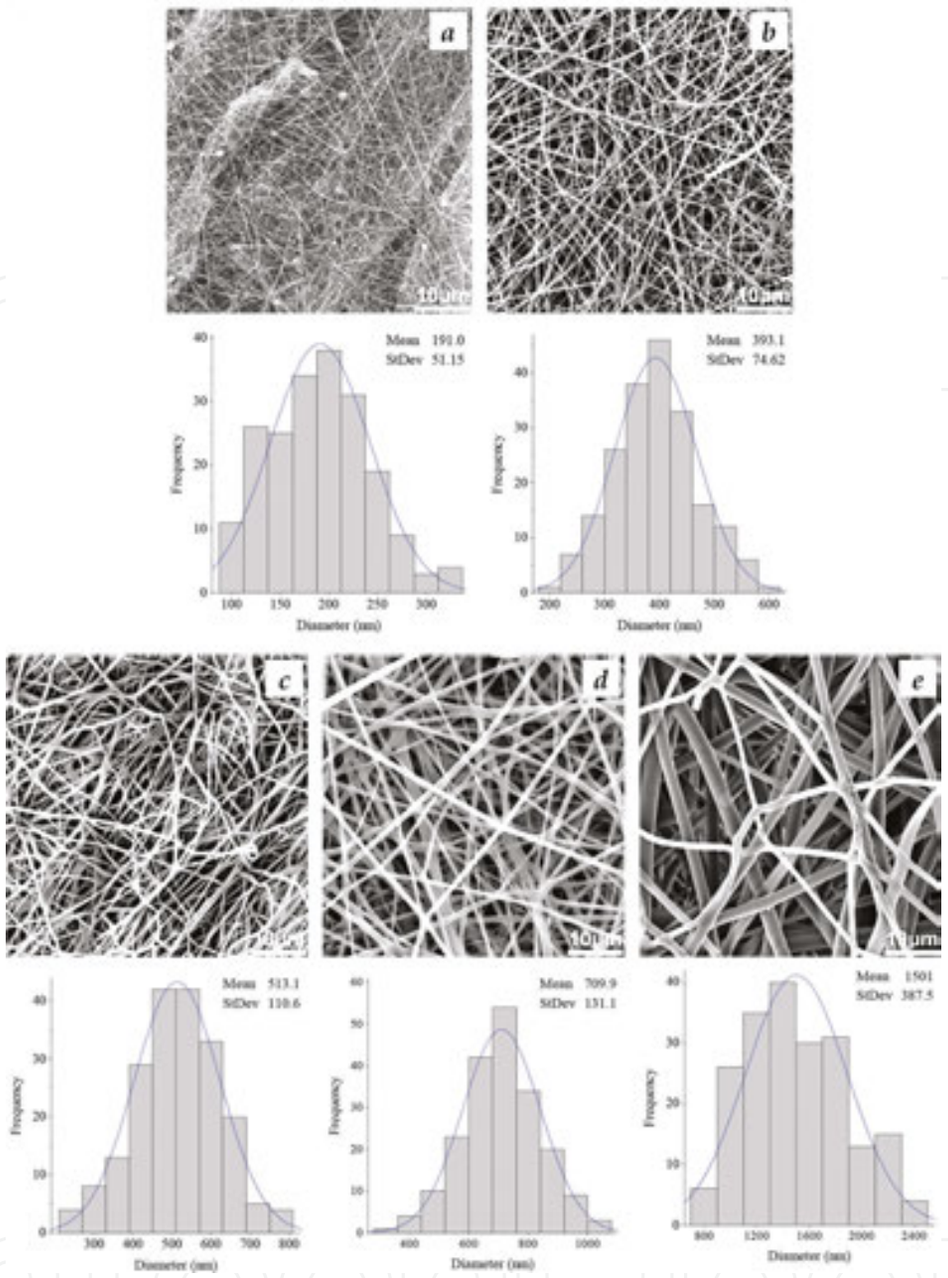

Figure 5. SEM micrographs and diameter distribution of electrospun fibres produced by needleless electrospinning with silk fibroin solution at various concentrations : (a) 6 wt%; (b) 8 wt%; (c) 10 wt%; (d) 12 wt%; (e) 14 wt% (magnification 5×).

In this study, the concentration of the silk solution played an important role in the spinnability of the needleless system. At low concentrations of spinning solution, non-fibrous formations were produced instead of nanofibres with beads. It is possible that Taylor cones are created in needleless electrospinning by picking up the spinning solution covering the surrounding spinning electrode [5, 14]. In low-concentration spinning solutions, the viscosity of the solution is also generally low. Such solutions cannot be loaded on the surface of the spinning electrode because of their lack of viscosity. When Taylor cones do not form on the surface of an electrode, the electrospinning process results in non-fibrous formations [24]. It was observed that no

fibres were formed when a silk fibroin concentration of less than 6 wt% was used for this spinning condition. Although a silk fibroin solution with a 6 wt% concentration can spin into nanofibres with the needleless system, the spinning solution still has a low viscosity. Therefore, some droplets were observed on the obtained fibre sheet. A further increase in the concentration of silk fibroin up to 8 wt% resulted in continuous nanofibres and the droplets disappeared. This was due to the fact that there were sufficient molecular chain entanglements in the polymer solution to prevent the breakup of the electrically driven jet and to allow the electrostatic stresses to further elongate the jet to form fibres. Therefore, the silk fibroin solution required a concentration of at least 8 wt% to produce continuous silk fibres with a nanometre diameter under the experimental conditions of the present study.

In addition to affecting the fibre morphology and spinnability, the concentration of the spinning solution also influenced the spinning performance (see **Figure 6**). Under the same processing parameters, the spinning performance increased constantly from 0.22 to 2.05 g/min/m when the silk fibroin concentration increased from 6 to 14 wt%. The reason for this was the high solution viscosity, which facilitated jet/filament formation.

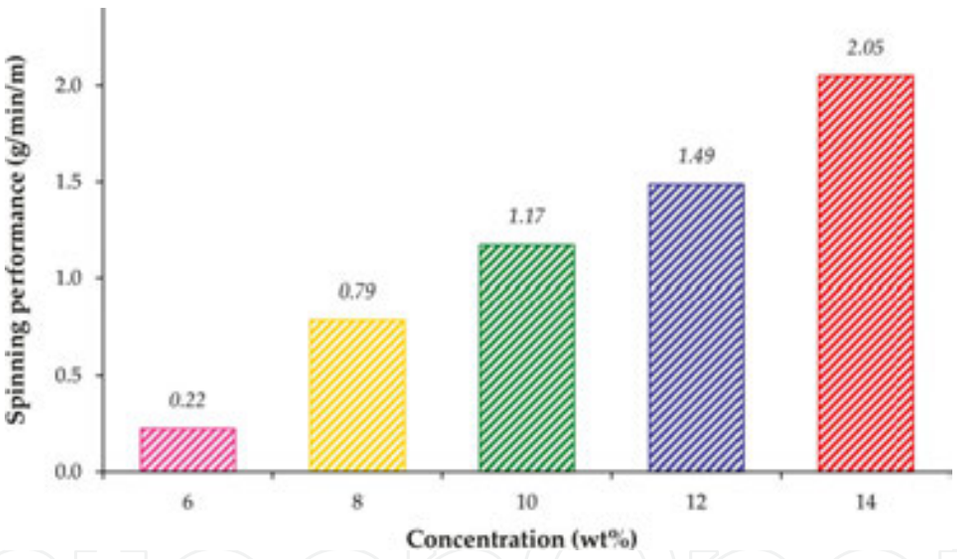

Figure 6. The effect of silk fibroin concentration on the spinning performance of the process.

3.2.2. The effect of applied voltage

The applied voltage is a very important parameter with regard to the formation of jets in electrospinning systems because a high voltage is used to create an electrically charged jet in a polymer solution [15, 18]. In order to evaluate the effect of the applied voltage on the electrospinning process, a spinning solution with a concentration of 12 wt% was electrospun at a voltage between 30 and 60 kV. SEM micrographs of the obtained fibre and their diameter distributions at the different voltages are shown in **Figure 7**. In the present study, the critical voltage required to initiate silk nanofibres in the needleless electrospinning system was higher than in the needle system [24]. When the silk fibroin solution was charged with an electric

voltage higher than 26 kV, a number of jets were generated from the surface of the spinning electrode. The results showed that increasing the applied voltage produced an effect on the fibre diameter, but the concentration of silk fibroin solution appeared to have a greater effect on the fibre diameter than did the applied voltage. With an applied voltage increase from 30 to 60 kV, the average fibre diameter decreased from 940 to 710 nm, respectively.

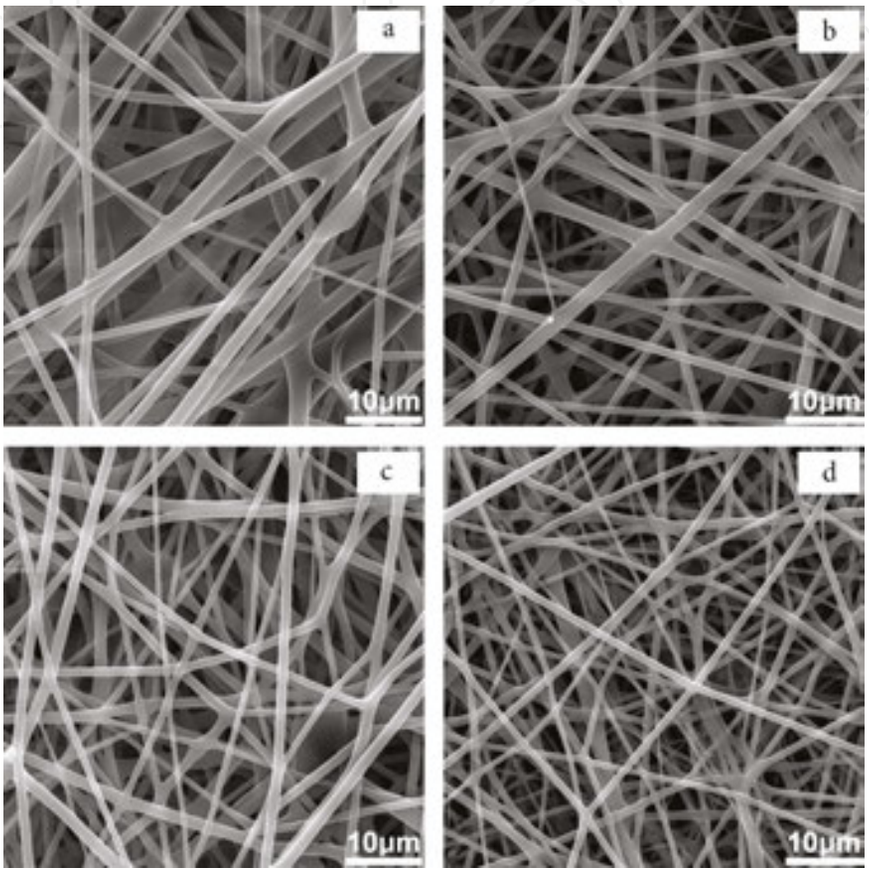

Figure 7. SEM micrographs and diameter distribution of electrospun fibres prepared by needleless electrospinning from silk fibroin 12 wt% at various applied voltages: (a) 30 kV; (b) 40 kV; (c) 50 kV; (d) 60 kV (SEM magnification 5×).

However, the spinning performance of the electrospinning process was influenced by the applied voltage and polymer concentration; it changed from 0.25 to 1.49 g/min/m, when the voltage was increased from 30 to 60 kV (**Figure 8**). As the electric field was the main driving force initiating the formation of Taylor cones and jets from the surface of the solution, increasing the electric voltage increased the electrostatic force on the polymer jet, which favoured further elongation of the jet and the formation of smaller fibres.

In contrast, the electric field also functioned to overcome the frictional forces that acted within the moving polymer solution and to accelerate filament movement towards the collector electrode. It is easier to generate solution jets at higher applied voltage in a polymer solution charged by a stronger electric field, because a larger amount of solution is removed from the surface of the solution, thereby improving the spinning performance of the process [12, 15, 17].

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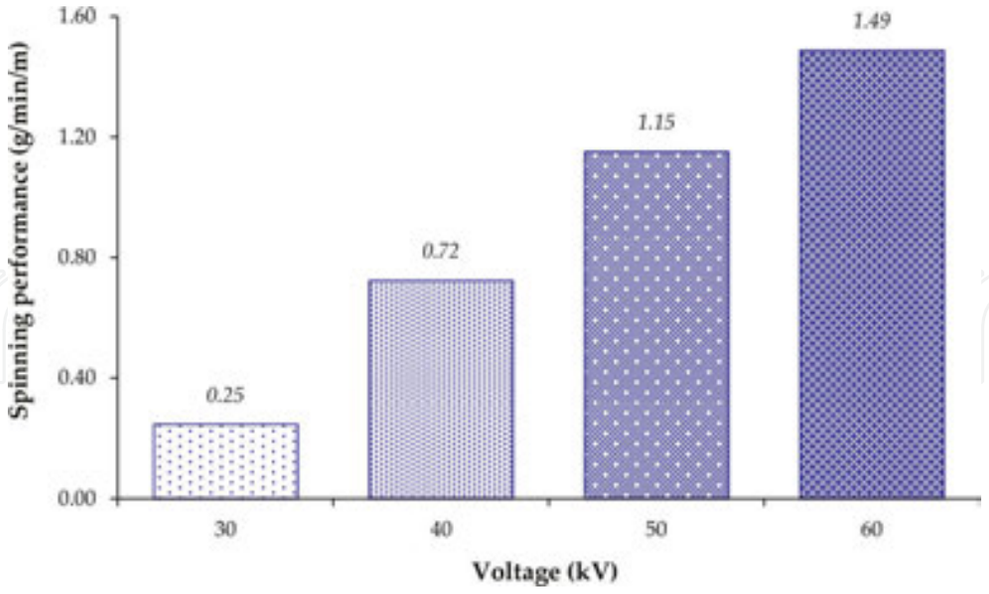

Figure 8. The effect of applied voltage on the spinning performance of the process.

3.2.3. The effect of distance between electrodes

In order to study the effect of spinning distance on the morphology of the obtained electrospun fibres and the spinning performance of the process, spinning solutions with a concentration of 12 wt% were electrospun at a high voltage of 60 kV. Electrospinning was carried out at a distance of 100, 125 and 150 mm. SEM micrographs of the resulting fibres and their distributions at the different spinning distances are shown in **Figure 9**. It was shown that an increase in spinning distance had a less significant effect on the average fibre diameter, but produced an effect on the spinning performance of the electrospinning process. With an increase in the distance from 100 to 150 mm, the average fibre diameter decreased from 710 to 647 nm, and the spinning performance of the electrospinning process changed from 1.49 to 0.70 g/min/m when the distance was increased (**Figure 10**). The distance between the spinneret and the collector is a key factor in determining the morphology of fibres and the spinning performances that are produced. It is suggested that increasing the distance has the same effect as decreasing the applied voltage, and that this will lead to a decrease in the field strength. As the electric field is the main driving force in initiation of the formation of jets from the surface of the solution, decreasing the electric voltage will decrease the electrostatic force on the polymer jet, resulting in a decrease of the spinning performance of the process. In other circumstances, increasing the distance results in a decrease in the average fibre diameter. As mentioned previously, jet elongation and thinning only occurs while the jet is in flight and still a fluid. This elongation occurs as a result of charge repulsion between ions in the solution combined with a net pull towards the collector. While in flight, the polymer solution solidifies as the solvent evaporates from the surface, forming polymer fibres. Therefore, increasing the spinning distance will increase the time for thinning to occur, and, provided the polymer is not yet solid, the fibre diameter will be reduced [22].

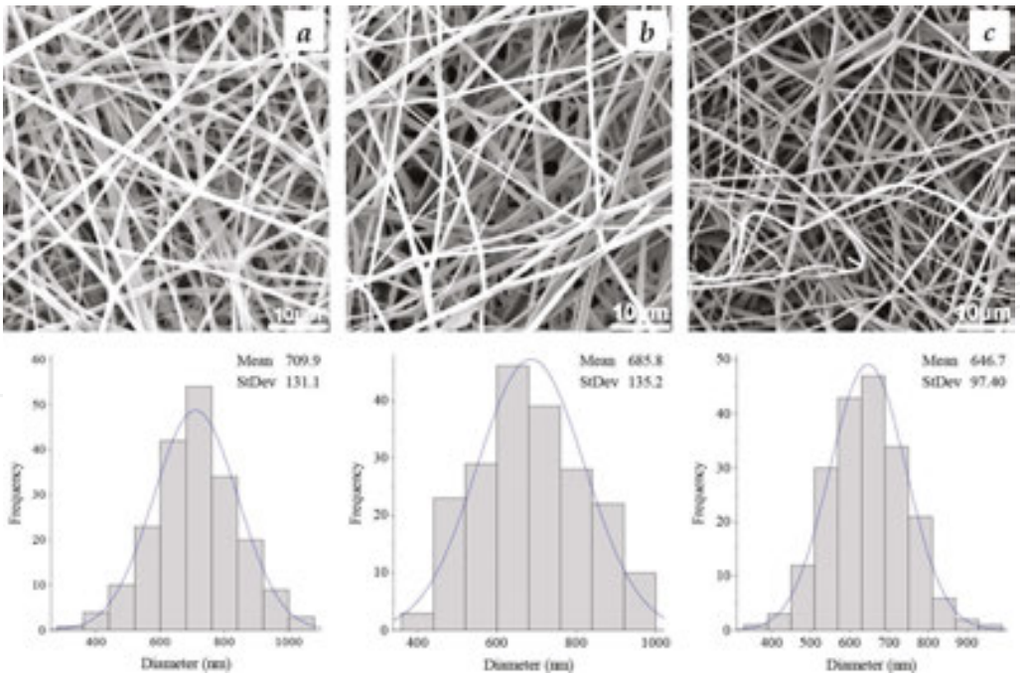

Figure 9. SEM micrographs and diameter distribution of electrospun fibres prepared by needleless electrospinning from silk fibroin 12 wt% at different spinning distance: (a) 100 mm; (b) 125 mm; (c) 150 mm (SEM magnification 5×).

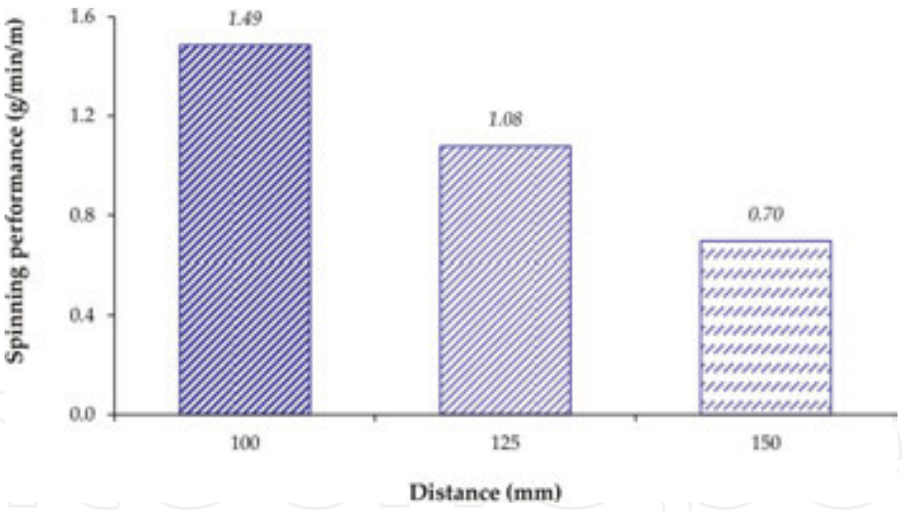

Figure 10. The effects of spinning distance on the spinning performance of the process.

3.3. *In vitro* cell interaction with MG-63 osteoblasts

The viability of cells seeded on the fibre sheets was measured by the MTT test during culture. The results are shown in **Figure 11**, and it can be seen that the tested materials supported osteoblast proliferation during 2 weeks of culture. The proliferation rate of MG-63 osteoblasts increased after 7 days of culture, whereas the absorbance increased. The highest proliferation rate was found between days 7 and 14.

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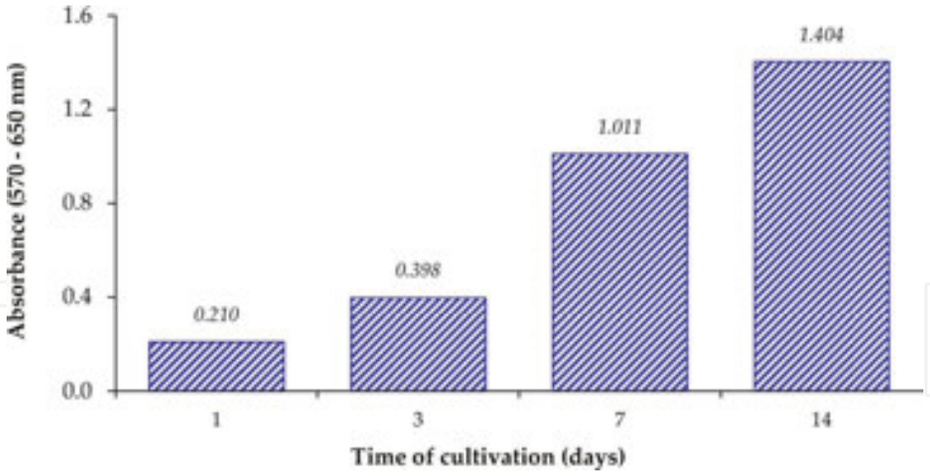

Figure 11. Cell viability measured by MTT test after culture with MG-63 osteoblasts.

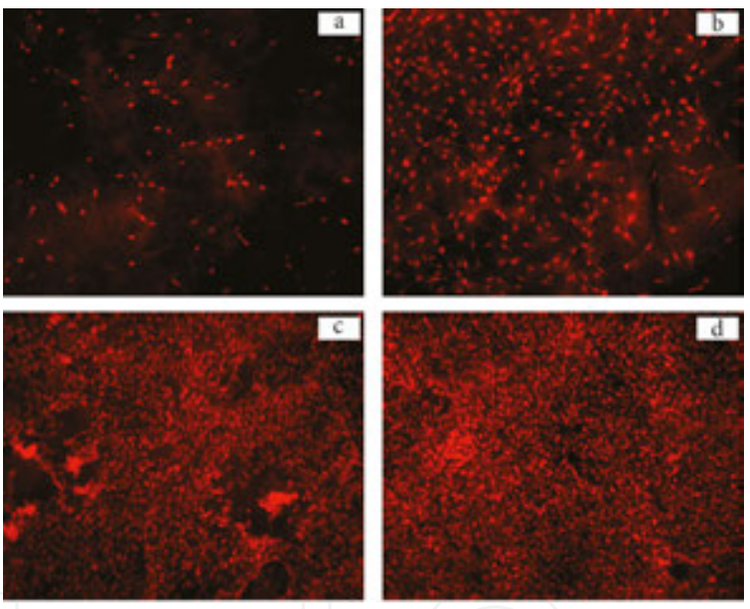

Figure 12. Fluorescence microscopy of MG-63 osteoblasts stained with propidium iodide during cell culture: (a) 1 day; (b) 3 days; (c) 7 days; (d) 14 days (magnification 100×).

As shown in **Figure 12**, the cells adhered and proliferated well on the surface of the fibre sheets during the time of culture. Fluorescence microscopy pictures confirmed the MTT test results that were obtained in the previous experiment. This showed a very high biocompatibility between silk fibroin and MG-63 osteoblasts.

The SEM micrographs from the scanning electron microscopy were in accordance with the fluorescence microscopy results, as well as the MTT test. As shown in **Figure 13**, the tested materials showed good adhesion when evaluated 1 day after seeding of osteoblasts onto the fibre sheets. During the 2 weeks of the experiment, the cells proliferated through the surface of the fibre sheets and covered most of their surface, and single cells can be distinguished. The

results were confirmed by viability measurement with the MTT test and fluorescence microscopy, as well as scanning electron microscopy.

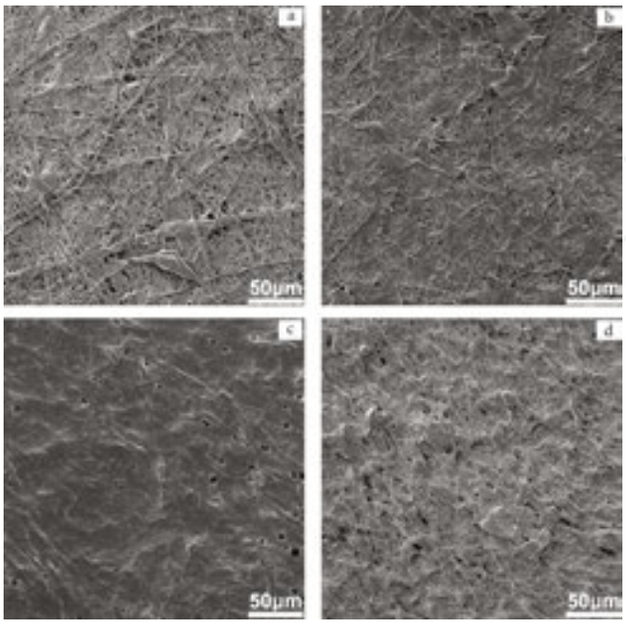

Figure 13. SEM micrographs of the fibre sheets after cells were cultured with MG-63 osteoblasts: (a) 1 day; (b) 3 days; (c) 7 days; (d) 14 days (SEM magnification 1×).

4. Conclusion

Silk fibroin nanofibres were electrospun with a needleless electrospinning method, focusing on the effect of the processing parameters on the morphology of the obtained fibres and the spinning performance of the electrospinning process. The use of formic acid-calcium chloride as the solvent for silk fibroin dissolution has the advantage of being simple in an operation. A solvent system consisting of formic acid and calcium chloride directly dissolved silk fibroin at room temperature. The weight ratio of 1:0.25 (w/w) of silk fibres to calcium chloride appears to be suitable for the dissolution of silk fibroin in formic acid. Thus, dissolution of silk fibroin in formic acid and calcium chloride could potentially be employed in the preparation of spinning solution for a large-scale production of silk nanofibres with a needleless electrospinning method.

In a needleless electrospinning process of silk fibroin in this solvent system, the concentration of the silk solution played an important role in the spinnability. Concentrations of silk fibroin ranging from 8 to 12 wt% appear to be suitable for the preparation of silk fibroin nanofibres with needleless electrospinning. Furthermore, increasing the concentration of the silk fibroin solution improved the spinning ability and the spinning performance of the electrospinning process. An increase in the applied voltage also enhanced the spinning performance of the process; however, an increase in the applied voltage had little effect on the reduction of the

diameter of silk fibroin electrospun fibres. In contrast, the variation of spinning distance in the spinning process affected the spinning performance. The spinning performance of the process was decreased when the spinning distance was increased. Although the electrospinning technique and dissolution method are different from those used in previous studies, the results from *in vitro* tests with MG-63 osteoblasts showed very high biocompatibility with regard to silk fibroin electrospun fibre sheets. The fibre sheets were capable of promoting adhesion, spreading and proliferation. It can be assumed that the electrospun fibre sheet is a promising material for biomedical applications, such as bone-tissue engineering.

Acknowledgements

This work was supported by the Technical University of Liberec, Faculty of Textile Engineering, Czech Republic, and the Ministry of Education, Youth and Sports as part of Project LO1201 targeted support from the programme 'Národní program udržitelnosti I'. The authors also thank Rajamangala University of Technology Phra Nakhon, Thailand, for providing the first author with a scholarship.

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